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Serum Factors Induce C-*fos* Expression and Rapid Cell Proliferation in Adolescent But Not in Infant Rat Proximal Tubule Cells

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ABSTRACT. Kidney epithelial cells in short-term primary culture have been studied with regard to proliferative rate and expression on the c-*fos* protooncogene. The experiments were performed on subconfluent renal proximal tubule cells isolated from infant and adolescent rats. Proliferation was determined by ^{3}H-thymidine autoradiography and nuclear content of c-*fos* protein by semiquantitative immunofluorescence. The basal proliferative rates in infant and adolescent renal proximal tubule cells were the same after 48 h of primary culture in Dulbecco's modified Eagle's medium with 10% FCS. Serum deprivation for 24 h caused a significant growth inhibition in both infant and adolescent cells. C-*fos* was expressed to the same extent in infant and adolescent serum-deprived cells. The rapid response to the addition of serum was markedly different in infant and adolescent cells. In adolescent cells, addition of serum led to a transient significant increase in the nuclear expression of c-*fos* protein, reaching a peak at 60 min. No increase in c-*fos* was seen in infant cells. In adolescent cells, the rate of proliferation increased 11-fold and ^{3}H-thymidine labeling index reached 26.7 ± 4.3%. In infant cells, the proliferative response to serum addition was significantly lower; the labeling index reached only 4.2 ± 1.2%. It could be excluded that the attenuated response in infant cells was due to cell death or impaired metabolic function. The results imply that the principles of growth regulation change postnatally. (*Pediatr Res* 29: 263–267, 1991)

Abbreviations

D, serum-deprived cells, cultured in the absence of serum for 24 h
DS, serum-deprived cells to which serum was added at the time of experiment
S, cells cultured in the presence of serum
DMEM, Dulbecco's modified Eagle's medium
LI, labeling index
RPTC, renal proximal tubule cells

The protooncogenes regulate growth and differentiation (reviews 1–3). The so-called immediate early response genes (4–8, review 9) are expressed within 1 h after stimulation of cells with growth factors. These genes code for transcription factors, some of which are necessary for the proliferative response (10, 11). The c-*fos* gene is an example of the immediate early response genes and is a well-characterized member of the protooncogene family (reviews 12–14). It is activated within minutes after the cell is stimulated with growth factors and participates in the regulation of proliferation (4–6, 11).

Animal studies have indicated that protooncogenes play an important role during embryonic development (2, 15, 16). Changes in the expression of some protooncogenes have also been observed in the pre- and perinatal period (15, 17–19). The role of protooncogenes for postnatal growth and maturation is not well known.

This study describes how renal epithelial cells from infant (10-d-old) and adolescent (40-d-old) rats can be used to study postnatal growth regulation. We have previously shown that RPTC obtained from the outermost renal cortex maintain transport properties (20–23), hormonal responsiveness (24), general morphologic characteristics (25), and similar proliferative rates (26) as found *in vivo* during the first few days in primary culture. The postnatal growth of the rat kidney is most rapid between 10 and 40 d of age (26, 27). The factors regulating growth during this period are not well defined, but data from whole animal studies have indicated that they might change during postnatal life (27, 28). Cells were isolated from 10- and 40-d-old rats because previous studies have shown that their basal proliferative rates are similar (26, 27).

In this study, serum-deprived RPTC were stimulated by the addition of FCS. The presence of c-*fos* protein in the nuclei was determined semiquantitatively with immunofluorescence and the proliferative rate was determined by ^{3}H-thymidine autoradiography. We found that cells from infant and adolescent rats responded differently with regard to the expression of c-*fos* protein and with regard to the proliferative response. The findings suggest that principles of growth regulation may change postnatally.

MATERIALS AND METHODS

Proximal Tubule Primary Cultures. Preparation of proximal tubule suspensions for primary culture has been described in detail previously (21). Briefly, infant (10-d-old) and adolescent (40-d-old) Sprague-Dawley rats were anesthetized with Mebumal 5 mg/100 g body weight intraperitoneally (ACO, Solna, Sweden) and the kidneys perfused with a sterile modified Hank's balanced salt solution with 0.05% collagenase (type 1; Sigma Chemical Co., St. Louis, MO). The outer 150 μm of the renal cortex was removed using a Stadie-Riggs microtome and incubated in the collagenase perfusion solution for 15 min. After a series of

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centrifugations, the tubule suspension was plated onto sterile Bürker slides or glass coverslips (Chance Propper Ltd., Great Britain). The cells were cultured in modified DMEM (Gibco, Paisley, Scotland) with 10% FCS (Gibco), in a water-jacketed incubator (Flow Laboratories, Meckenheim bei Bonn, Germany), at 37°C with 5% CO_2 and 95% air. The modified DMEM contained 20 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 24 mM $NaHCO_3$, 50.000 IU/L penicillin, and 50 mg/L streptomycin (Flow Laboratories); pH set to 7.4 at 37°C and 5% CO_2. Medium was changed after 1 d of culture. In studies of serum-deprived cells, the medium was carefully rinsed and replaced by DMEM after 24 h in culture. Experiments were performed after 48 or 72 h in culture depending upon protocols.

The cells grew in colonies. Central confluent cells in the colonies have a lower proliferative rate than subconfluent peripheral cells, probably due to density-dependent growth inhibition or contact inhibition (23). In the present work, we have studied growth regulation and nuclear c-*fos* expression in subconfluent cells defined as the two outermost cell layers in each colony.

In all experiments, cells were cultured in 10% FCS during the first 24 h. The cells were then either cultured another 24 h in the presence of FCS (S), serum-deprived for 24 h (D), or serum-deprived for 24 h and then returned to serum-containing media at the time of experiment (DS). After serum deprivation, no cell loss or cytoplasmic blebbing was observed. Serum-deprived cells also maintain functional integrity assessed by determinations of intracellular K/Na and pH. The cytoplasmic K/Na ratio studied by electron probe microanalysis is 11.1 ± 1.7 (n = 7) in adolescent RPTC and 9.6 ± 0.1 (n = 5) in RPTC from 11- to 12-d rats after 24 h serum deprivation and 2- to 3-d in primary culture. This indicates that the cells are capable of maintaining normal electrolyte gradients across the cell membrane. (20–22; SH Larsson, unpublished results). Furthermore, intracellular pH in serum-deprived infant and adolescent cells were not significantly different [7.29 ± 0.05 (n = 10) *versus* 7.31 ± 0.03 (n = 4)] and were well within the physiologic range for the proximal tubule (23, 29).

^{3}H-Thymidine Autoradiography. S and D cells were pulsed for 6 h with ^{3}H-thymidine (1–2 μCi/mL) (Amersham, Buckinghamshire, England). The response to serum addition (DS) was studied after a 24-h pulse, assuming the cells needed 12–18 h to move from G0 of the cell cycle into S phase. After labeling, the cells were fixed (ethanol:formaldehyde:acetic acid, 72:10:18) and stained with orcein as described previously (23). The coverslips were dipped in a photographic emulsion (Kodak NTB2) and exposed at 4°C for 72 h. They were developed with Kodak Dental x-ray developer and fixed. LI was calculated as the percentage of cells with >50 silver granules over the nucleus. The cells in eight to 10 colonies were counted, *i.e.* >600 cells/animal and protocol. LI for adolescent RPTC under S and D conditions has previously been published (23).

Semiquantitation of c-fos Content. Immunocytochemical staining. The nuclear expression of the c-*fos* protein was determined with immunocytochemical staining in accordance with the indirect immunofluorescence technique of Coons (30). Two sets of antibodies were used. In most experiments, we utilized the xfos-3 antibodies generously supplied by Dr. G. Evan (31). Control experiments were performed using affinity purified sheep anti-*fos* antibodies DCP 821 (Cambridge Research Biochemicals, Cambridge, England). Both xfos-3 and DCP 821 are directed against amino acid residues 2–17 of the p55$^{c\text{-}fos}$ protein.

Nuclear c-*fos* content was determined in RPTC from infant and adolescent rats after growth stimulation (DS). The effect of growth stimulation was studied at different intervals after serum addition (15, 30, 60, 120, and 240 min). At the end of the experiment, the cells were fixed at 4°C in 4% paraformaldehyde in 0.1 M PBS for 15 min. After fixation, the cells were rinsed three times in PBS and incubated overnight at 4°C with anti-*fos* antiserum at a dilution of 1:500. The cells exposed to xfos-3 were then rinsed in PBS and incubated for 1 h with swine anti-rabbit antibodies conjugated to rhodamin (Dako-Ig a/s, Glostrup, Denmark). The antibodies were diluted 1:100 and incubation took place in the dark at room temperature. Cells exposed to DCP 821 antiserum were incubated with FITC-conjugated anti-sheep antisera in an identical protocol. All antisera were diluted in PBS with 0.5% Triton x-100. After a final rinse in PBS, the slides were mounted in glycerine and PBS (1:1). In experiments where the two anti-*fos* antisera were used in parallel, they consistently gave similar results.

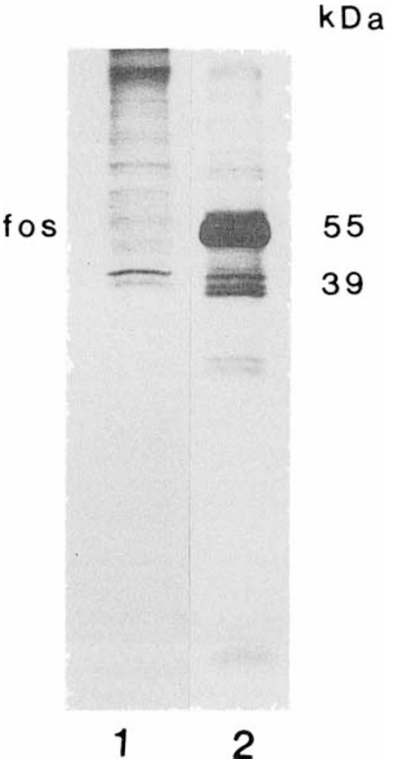

Fig. 1. Precipitation of c-*fos* protein by xfos-3 antibodies. *Lane 1*, immunoprecipitation of c-*fos* protein from Swiss 3T3 cells that express low levels of c-*fos*. *Lane 2*, immunoprecipitation of c-*fos* protein from cells of the Swiss 3T3 subclone MF-2 that express mouse c-*fos* from a transfected plasmid (p$^{76/21}$) (32). Sizes are shown in kD. Markedly larger xfos-3 antigenic activity was found in transfected cells compared with controls. Method: 5 × 10^6 cells were preincubated with methionine-free DMEM for 30 min. Thereafter, they were incubated with 1 mL of methionine-free DMEM containing 200 μCi of ^{35}S-methionine (Amersham) for 30 min at 37°C. The cells were washed three times in PBS and lysed with 1% aprotinin in RIPA buffer (50 mM NaCl, 25 mM Tris HCl pH 8.2, 0.1% Na azide, 0.5% Na deoxycholate, 0.5% NP40, 0.1% Na dodecyl sulfate). Cell lysates were spun at 10 000 rpm for 10 min at 4°C. Supernatants were preadsorbed with normal rabbit Ig-sepharose and were thereafter incubated with 1 μL of xfos-3 antibodies for 30 min on ice. Immunecomplexes were collected with protein A-sepharose. Precipitates were analyzed on 12% polyacrylamide gels (33) and the gel was processed for fluorography (34). Molecular weights were determined relative to Rainbow protein molecular weight markers (Amersham).

Quantitative fluorescence microscopy. The c-*fos* antigenic activity in individual nuclei of the proximal tubule cells was measured as the nuclear content of the fluorescent second antibody, *i.e.* the intensity of rhodamine (xfos-3) or FITC (DCP 821) fluorescence. The fluorescence intensity was quantitated using computerized image analysis. The cells were studied in a dark-field microscope with epi-illumination using a primary magnification of 63× (Zeiss, Oberkochen, Germany). The fluorescence microscope was equipped with a silicon-intensified target video camera connected to an image analysis system (IBAS, Kontron-Zeiss, München, Germany). The sample was only illuminated

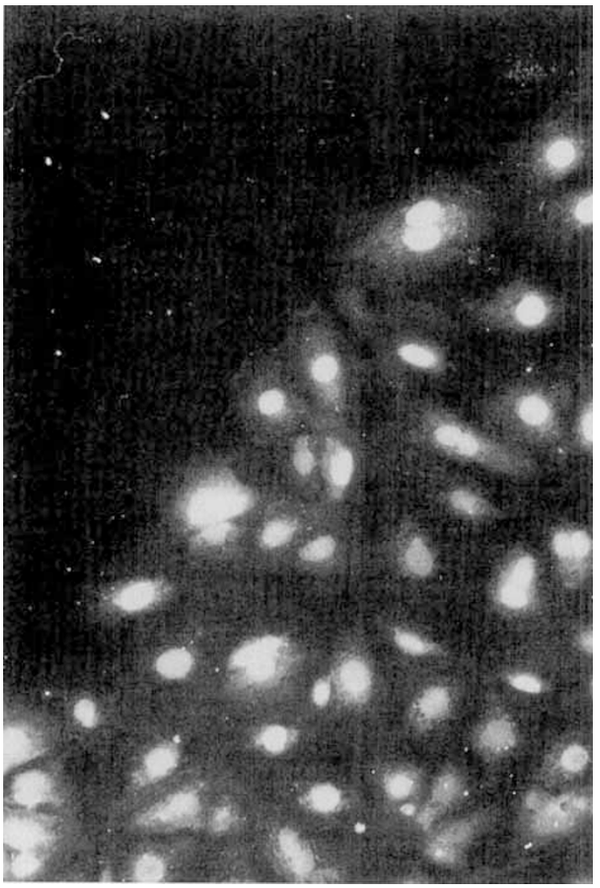

Fig. 2. C-*fos*-specific fluorescence in cells from infant rats after 24 h serum deprivation. Note the nuclear distribution of the fluorescence.

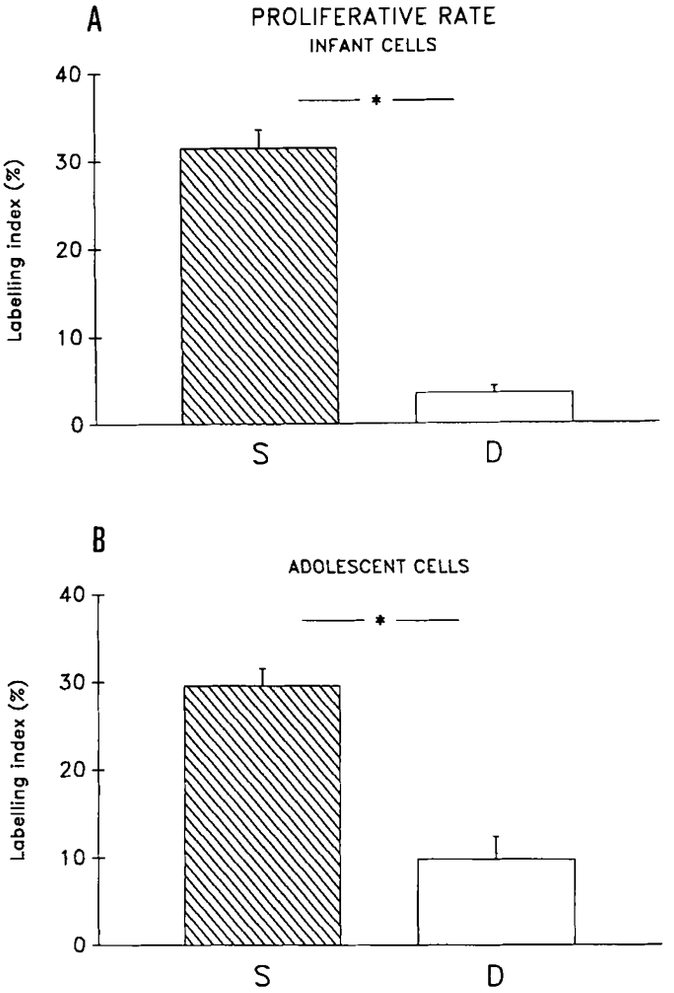

Fig. 3. LI in infant (*A*) and adolescent cells (*B*) cultured in the presence of serum (*S, hatched bar*) or deprived of serum for 24 h (*D, open bar*). Autoradiography was performed after a 6-h pulse of ^{3}H-thymidine (1–2 μCi/mL). The experiments were performed after 48 h in primary culture. Twenty-four hours of serum deprivation resulted in a significant decrease in LI in both infant (90 ± 2%) and adolescent cells (69 ± 8%); the responses were, however, significantly different ($*p < 0.05$). The experiments were performed in parallel, but the adolescent values have been published previously (23).

during focusing and digitizing of the image to avoid fading. Each image was converted into an array of 512 × 744 picture elements (pixels), corresponding to 200 × 300 μm, with a resolution of 256 gray levels for each pixel.

In each image, the nuclear profiles were encircled by careful tracing with a cursor. An area outside the cell colony was also encircled for determination of background light intensity. After identification of the selected regions, the median values of the pixel gray levels in each cell nucleus and in the background were measured. The distribution in each selected area was generally gaussian. The nuclear values obtained were corrected for possible variation in the camera gain by dividing the nuclear gray levels with the background gray level. Samples in which the primary antibody was omitted served as negative controls to determine the nonspecific fluorescence in the cell nuclei. The nonspecific fluorescence was subtracted from each nuclear determination. The two-dimensional areas of measured nuclei were quantitated using a standard procedure of the image analysis system to evaluate whether or not changes in apparent c-*fos* activity were related to changes in nuclear size. Cells in two separate regions from each of six colonies, *i.e.* 50–90 cells, were studied for each observation.

Results are expressed as means ± SEM, unless otherwise stated. Group comparisons were based on parametric paired or non-paired *t* statistics and timecourse changes evaluated by variance analysis.

RESULTS

The specificity of the c-*fos* protein determinations was confirmed by three findings. First, transfection of 3T3 fibroblasts with a c-*fos* containing plasmid ($p^{76/21}$) markedly increased the xfos-3 specific immunoprecipitation (Fig. 1). Second, the immunofluorescent stain in the cultured proximal tubule cells was characteristically concentrated to the cell nucleus (Fig. 2) (17, 35). Third, experiments with two different c-*fos*-specific antisera showed a similar response pattern in all experimental conditions (see below).

Subconfluent proximal tubule cells from infant and adolescent rats had the same proliferative rate after 2 d in primary culture (LI = 31.5 ± 2.1 *versus* 29.6 ± 2.0%, NS; n = 5, 6). Serum deprivation for 24 h decreased the growth rate significantly in both infant (90 ± 2%) and adolescent cells (69 ± 8%) (Fig. 3). After 24 h of serum deprivation, no difference in c-*fos* activity was found between infant and adolescent cells (not shown).

When serum-deprived adolescent cells were exposed to 10% FCS, a rapid transient increase in c-*fos* activity was seen, showing a peak at 60 min after serum addition (Fig. 4). In several experiments, a late second elevation was noted at 240 min, which may be attributed to *fos*-related antigens (36). The results were reproduced in five experiments with xfos-3 antibodies and in a control experiment with DCP 821 [D: 4.9 ± 0.2; DS(30 min): 5.6 ± 0.3; DS(60 min): 7.2 ± 0.2 relative fluorescence units]. After serum addition, LI in adolescent cells increased from 2.4 ± 0.8 to 26.7 ± 4.3% (n = 4) (Fig. 5).

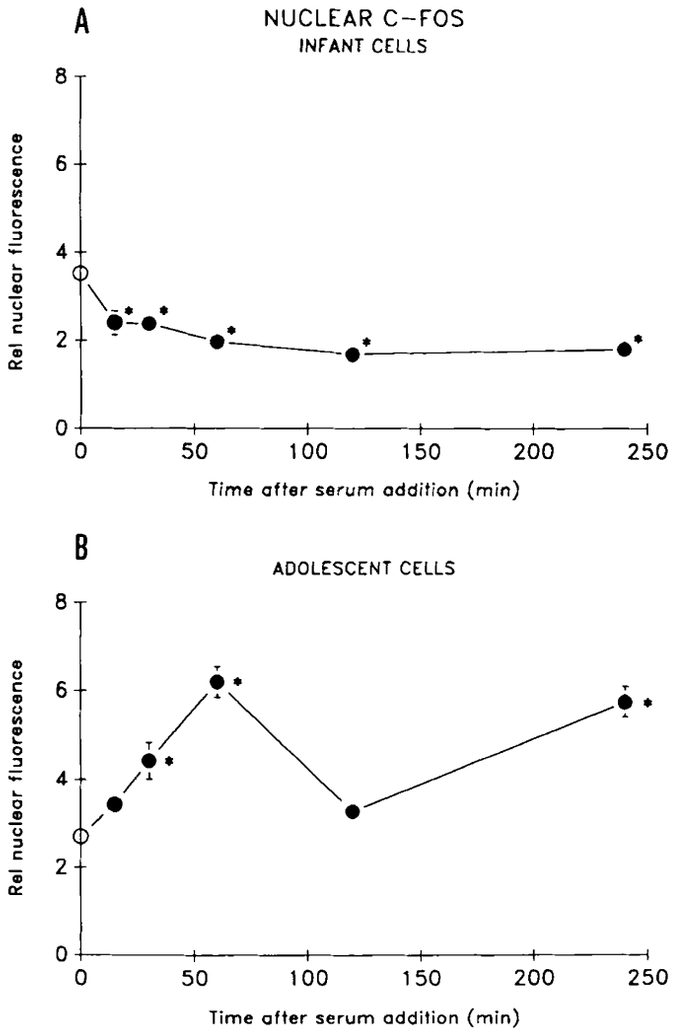

Fig. 4. Change in nuclear c-*fos* expression after addition of serum (DS) to serum-deprived cells (D, 24 h). *A*, in infant cells, a decrease in c-*fos* content was noted at 15–30 min and the level remained low throughout the experiment. * Value significantly lower than during D condition ($p < 0.05$). *B*, in adolescent cells, a transient increase in nuclear c-*fos* fluorescence was seen, showing a peak at 60 min. A second elevation was also seen in several experiments at 240 min. * Value significantly higher than during D condition ($p < 0.05$).

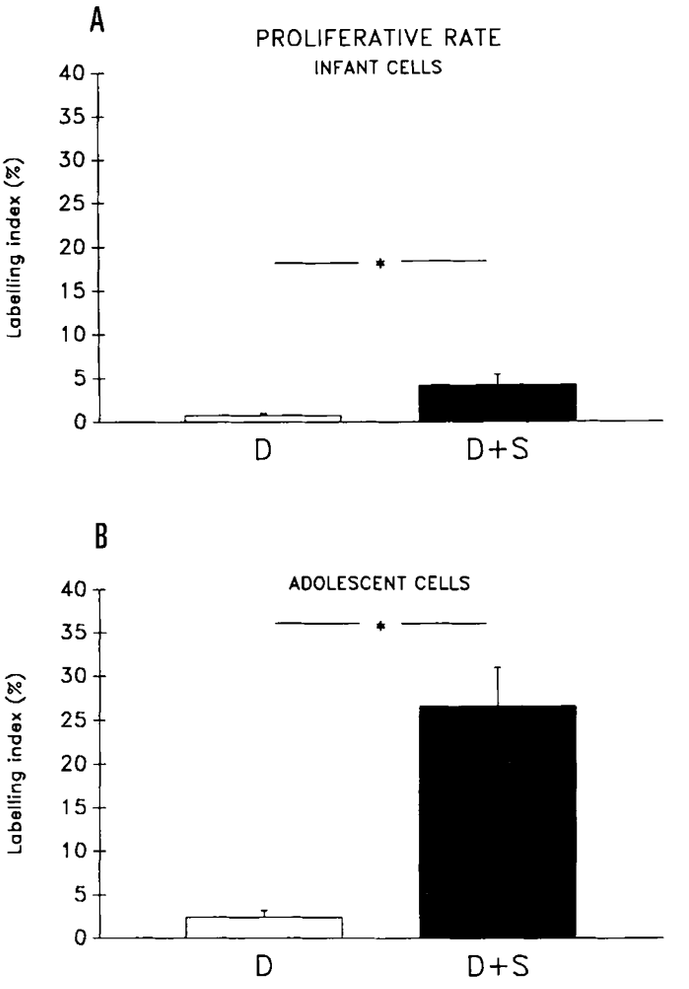

Fig. 5. LI in infant (*A*) and adolescent cells (*B*) after 24 h serum deprivation. The cells were labeled with ^{3}H-thymidine (1–2 μCi/mL) during the subsequent 24 h either in the absence of serum (*D, open bar*) or after addition of 10% FCS to the medium (*DS, filled bar*). The cells were thus fixed and the autoradiography performed after 72 h in culture. (* $p < 0.05$).

Table 1. *Nuclear size determined as surface area of nuclear profiles from microscopic image (μm²)**

Age (d)	Protocol D	Protocol DS
10	147 ± 12	140 ± 15
40	129 ± 11	150 ± 18
	NS	NS

* The values were determined in cultured RPTC from infant (10 d) and adolescent (40 d) animals during growth inhibition (D) and after growth stimulation (DS, 60 min). $n = 3$.

Serum-deprived infant cells showed a markedly different response to 10% FCS. Throughout a 4-h timecourse, no increase in c-*fos* activity was seen. Contrarily, a fall in c-*fos* activity was noted after 15–30 min exposure to 10% FCS (Fig. 4). The results were reproduced in four experiments with xfos-3 antibodies and in one control experiment with DCP 821. The proliferative response of infant cells also differed from that of adolescent cells. LI only increased from 0.7 ± 0.2 to 4.2 ± 1.2% after serum stimulation ($n = 5$) (Fig. 5). The differences in c-*fos* response between infant and adolescent cells cannot be explained by changes in nuclear size, estimated as surface area of nuclear profiles (Table 1).

DISCUSSION

It has been suggested that several members of the protooncogene family, including c-*fos*, will act to regulate cell growth (2, 3, 14). The results in this study are compatible with that hypothesis. In the adolescent RPTC, serum stimulation increased cell growth. This increase was preceded by an increase in the expression of c-*fos* product. In infant RPTC, there was no increase in the expression of the c-*fos* product and only a minimal increase in cell growth after serum stimulation (Figs. 4 and 5).

In adolescent RPTC, the addition of FCS to serum-deprived cells caused a significant transient increase in nuclear c-*fos* protein content with a peak at 60 min (Fig. 4). A similar rapid response to growth stimulation has been documented in fibroblasts (4–6). A detectable increase in c-*fos* mRNA is seen already at 10 min and is followed by an increase in c-*fos* protein synthesis, reaching a peak after 60 min. As in fibroblasts, the rapid and transient increase in the expression of the c-*fos* protein precedes an increase in cell growth in RPTC. The addition of FCS to serum-deprived adolescent RPTC was followed by an 11-fold increase in LI (Fig. 5).

In contrast to adolescent RPTC and fibroblasts, serum-deprived infant cells did not respond to serum stimulation (DS protocol) with increased c-*fos* expression (Fig. 4). The growth stimulatory effect of serum addition was also blunted in infant cells (Fig. 5).

Both infant and adolescent cells grew well in the presence of serum. Serum deprivation markedly inhibited proliferation in both groups. After 24 h of serum deprivation, both infant and adolescent cells were quiescent, showing a LI of only 0.7 ± 0.2 and 2.4 ± 0.8%, respectively, after a 24-h ^{3}H-thymidine pulse (Fig. 5). A fairly large fraction of adolescent cells reentered the cell cycle within 24 h after addition of serum. The reactivation of infant cells during this period was markedly attenuated. Similar differences between infant and adolescent RPTC have been observed with regard to epidermal growth factor and IGF-1 (SH Larsson, unpublished studies). It is unlikely that the blunted response to growth factors in infant cells is due to decreased viability. The cells remain intact on the culture support during the serum-deprivation period, maintaining a high K/Na ratio and normal regulation of intracellular pH (21–23, 29). There are a number of cell functions that might change during ontogeny and that could explain the blunted response to growth factors in infant cells. The abundance of growth factor receptors might be lower in infant cells (37, 38). Intracellular signal transduction systems that are coupled to the growth factor receptors might be immature in infant cells. These signal systems are likely to involve activation of protein kinase C (14, 39, 40). Ontogenic changes in the expression of protein kinase C activity have been documented in the CNS and in RPTC (41, 42). There might also be an age-dependent regulation of the c-*fos* gene (15, 17, 18). Future studies will be directed to differentiate between these possibilities.

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