Retinoic Acid Inhibition of Serum-induced c-fos Transcription in a Fibrosarcoma Cell Line

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ABSTRACT

We investigated the mechanism by which retinoic acid causes growth arrest and flat reversion of SSV-NRK, simian sarcoma virus-transformed normal rat kidney cells. Northern analysis revealed that both chronic (7 days) and acute (6 h) retinoic acid treatment of serum-stimulated SSV-NRK cells caused a 6-fold decrease in c-fos mRNA levels. In addition, nuclear run-on experiments showed that retinoic acid regulated c-fos expression in SSV-NRK cells at the transcriptional initiation level. Attenuation of c-fos transcription was equal in both retinoic acid-treated and control cells, and no increased c-fos mRNA turnover was detected in retinoic acid-treated cells. Furthermore, there was no observed change in the c-fos mRNA levels after only 30 min of retinoic acid treatment, suggesting that a mechanism involving the interruption of the signal transduction mechanism at the membrane level is unlikely. Because it has been shown that c-fos expression plays a pivotal role in mitogenesis of quiescent fibroblasts, we conclude that the retinoic acid-mediated down-regulation of c-fos expression is a mechanism for growth inhibition in SSV-NRK cells.

INTRODUCTION

In 1925 it was initially observed in rats that vitamin A deficiency leads to squamous metaplasia of mucous membranes and hyperkeratosis of the skin (1). Since then, RA, a physiologically active vitamin A derivative, has been shown to inhibit the proliferation of a broad range of transformed cell types (2-4), as well as to induce benign (terminal) differentiation in a group of transformed cell lines, including F9 murine teratocarcinoma (5), SMS-KCN human neuroblastoma (6), and HL-60 human promyelocytic leukemia (7). The mechanism by which RA mediates its multiple effects on transformed cells is not well understood.

RA has been shown to inhibit anchorage-independent growth of SSV-NRK, a rat fibroblast cell line transformed by simian sarcoma virus (8). Autocrine stimulation of these cells by p287, a homologue of platelet-derived growth factor encoded by v-sis, resulted in unregulated proliferation (9). Although constitutive expression of v-sis has been demonstrated to be essential for the transformed state of the cells (10), RA has been shown to have no effects on v-sis expression (8). Therefore, other mechanisms are probably involved in mediating the growth inhibition of the transformed cells by the retinoids.

A cascade of events occurs following mitogenic stimulation of fibroblasts by growth factors, leading eventually to cell division. Belonging to a group of immediate-early genes, c-fos plays a pivotal role in these events, allowing cells to pass from G0-G1 to the S phase of the cell cycle (11). Mitogenic stimulation of serum-starved fibroblasts by serum or growth factors such as platelet-derived growth factor is associated with a very rapid induction of c-fos (12, 13). When a plasmid expressing antisense c-fos RNA is introduced into such an experimental system to block c-fos expression, the cell division does not occur (14).

Furthermore, transformed cells expressing antisense c-fos undergo flat reversion suggestive of benign differentiation of the neoplastic cells (15). We observed that RA also caused flat reversion of SSV-NRK cells. Therefore, we hypothesize that RA affects the signal transduction pathway, in particular the expression of c-fos. In this paper, data will be presented showing down-regulation of c-fos transcription in serum-stimulated SSV-NRK cells by RA.

MATERIALS AND METHODS

Cell Culture. SSV-NRK cells (a gift from Dr. S. Aaronson) were grown in Dulbecco's medium enriched with 10% heat-inactivated calf serum. For treatment of cells, a stock of 4 × 10^7/ml all-trans-RA (Sigma, St. Louis, MO) was prepared in 100% ethanol, stored at -20°C, and diluted immediately before use. In control cultures, ethanol was added to a final concentration of 0.25%. Cycloheximide was added to 50 µg/ml where indicated. Cell growth kinetics were measured in triplicate in 35-mm plates seeded with 2 × 10^4 cells/plate and cell number was counted daily with a hemocytometer. Routine tests for Mycoplasma by the Hoechst staining method (16) were negative.

RNA Extraction. RNA was prepared by the method of Chirgwin et al. (17). Briefly, total cellular RNA was extracted from approximately 10^6 SSV-NRK cells. Cells were lysed with 6.7 M guanidine thiocyanate (Research Organics, Cleveland, OH) in the presence of 1 mM vanadyl ribonucleoside complex (Bethesda Research Laboratory, Gaithersburg, MD), a RNase inhibitor. The lysate was centrifuged over a cushion of 3.5 M CsCl. The pellet RNA is introduced into such an experimental system to

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'The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; SRE, serum response element; SRF, serum response factor; SSC, standard saline citrate; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; SSPE, saline-sodium phosphate-EDTA.

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in the latter solution at 65°C for 45 min. The filters were then exposed to X-ray film, using an intensifying screen at −70°C.

Nuclear Run-On Transcription. Run-on transcription was carried out as described by Mahajan and Thompson (22) with modifications. Nuclei were prepared from approximately 10⁷ cells, and in vitro transcription was initiated by adding to 10 μl nuclear suspension a 100-μl mixture of 0.5 M Tris-Cl, pH 7.8, 0.25 M NaCl, 1.75 M (NH₄)₂SO₄, 0.01 M EDTA, 0.2 M MgCl₂, 1 mg/ml heparin, and 100 μm each of ribonucleoside triphosphates containing 100 μCi [α-³²P]UTP (Du Pont NEN, Wilmington, DE) at 32°C. After incubation for 45 min, RNA was extracted with phenol/chloroform, and ethanol precipitated. Nuclear equivalents of labeled RNA from control and RA-treated cells were hybridized to M13 DNA probes containing either exon 1 or exons 3 and 4 of murine c-fos (23) that were immobilized on nitrocellulose filters by using the slot-blot apparatus (Schleicher & Schuell). Plasmid pTH1 (kindly provided by Dr. E. A. Thompson), which encodes SS RNA, was used as positive control. Prehybridization and hybridization were carried out in a buffer containing 50% formamide, 5×SSC, 0.2% SDS, 1 mg/ml sheared yeast DNA, and 50 μg/ml polyadenylic acid at 65°C for 2 and 40 h, respectively. Filters were washed sequentially with 2 × SSC at 65°C for 1 h, 2 × SSC containing 10 mg/ml RNase A (Boehringer Mannheim, Indianapolis, IN) at 37°C for 30 min, and 2 × SSC at 37°C for 1 h. The filters were then exposed to X-ray film as described above.

Materials. Ultrapurified cesium chloride was from Bethesda Research Laboratories. Agarose (Seakem LE) was supplied by FMC BioProducts (Rockland, ME). Polyadenylic acid was purchased from Boehringer Mannheim. Actinomycin D and cycloheximide were supplied by Merck Sharp & Dohme (West Point, PA) and Sigma, respectively. All other reagents were of the highest grade available and were purchased from standard sources.

RESULTS

Effects of RA on Cell Growth. SSV-NRK cells (7.5 × 10⁶) were grown on duplicate 60-mm Petri dishes in a series of dilutions of RA. The growth of these cells was inhibited by RA in a dose-dependent manner: 85% of controls at 10⁻² M RA, 70% at 10⁻³ M RA, and 60% at 10⁻⁴ M RA. Morphological changes suggestive of flat reversion were also observed in all RA-treated cultures, as early as 14 h in 10⁻⁴ M RA. Our results agree with published data that anchor-age-dependent growth of SSV-NRK cells was inhibited by RA at the median effective dose of 5 × 10⁻⁴ M (8).

Effects of RA on c-fos Expression. The expression of c-fos has been reported to be one of the earliest responses in fibroblasts after mitogenic stimulation (13). We assayed c-fos mRNA in response to serum stimulation in RA-treated cells. Cells were grown to confluence and serum starved in 0.5% FBS for 48 h. During this time, they divided extremely slowly and-treated cells makes the half-life estimate rather inaccurate. It is clear, however, that based on two independent experiments, the c-fos mRNA decay in RA-treated cells was not accelerated. Thus, RA does not appear to down-regulate the serum-induced c-fos mRNA level by increasing its turnover.

Figure 1. Northern blot analysis of c-fos (A) and v-sis (B) mRNA in RA-treated SSV-NRK cells. The cells were grown to confluence, serum starved, and serum stimulated as described in the text. Cells were treated with 10⁻⁴ M RA (Lane 1) or untreated (Lane 2). The sizes of c-fos and v-sis mRNA are 2.2 and 2.7 kilobases (kb), respectively. The same blots were stripped and rehybridized with a tubulin probe.

Half-Life of c-fos mRNA in RA-Treated Cells. Since RA may down-regulate the c-fos mRNA levels by increasing its degradation, we performed actinomycin D experiments to measure the rate of c-fos mRNA decay. SSV-NRK cells were grown to confluence, serum starved, and serum induced as described above. Immediately following a 30-min period of serum induction, actinomycin D at 30 μg/ml was added. The cells were harvested at the time of actinomycin D addition and at 15-min intervals thereafter. RA was added 6 h before actinomycin D and maintained in the cultures until harvesting. The decay of c-fos mRNA in the control was very rapid (Fig. 2), with a half-life of 12 min, which is in agreement with previous reports (24, 25). The half-life of c-fos mRNA in RA-treated cells was estimated to be 17.5 min. The low level expression of c-fos in the drug-treated cells makes the half-life estimate rather inaccurate. It is clear, however, that based on two independent experiments, the c-fos mRNA decay in RA-treated cells was not accelerated.

To determine the site of action of RA in down-regulating c-fos expression, we examined the level of transcription initiation and elongation. An alternative site of action of RA in down-regulating c-fos expression is at the level of transcription initiation and elongation. Therefore, we performed nuclear run-on experiments. SSV-NRK cells were grown to confluence in 10% FBS, serum starved for 48 h, and serum induced for 30 min with 20% FBS. Cells were grown in the presence of 10⁻³ M RA for 7 h before harvesting. Fig. 3 shows that the transcription of the first exon of c-fos in RA-treated cells was much reduced. From densitometric analyses, the intensity of the band corresponding to the first exon is decreased 4-fold in RA-treated cells as compared to control cells (after correcting for small differences in the SS RNA-positive controls). The difference between RA-treated and con-
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![Graph showing transcription initiation and elongation](image-url)

**Fig. 2.** Determination of the half-life of c-fos mRNA. Confluent, serum-starved cells were exposed to 10^{-5} M RA 5.5 h prior to serum stimulation. Twenty % FBS containing RA was then added and 30 min later 30 μg/ml actinomycin D was added. Cells were harvested at time 0, i.e., the time of actinomycin addition (Lane 1), and at 15-min intervals thereafter (Lanes 2, 3, 4, and 5). RNA was extracted and analyzed by Northern hybridization techniques. The 2.2-kilobase (kb) band is the c-fos mRNA. +RA, with RA treatment; -RA, without RA treatment. tub, tubulin.

![Graph showing expression of c-fos mRNA](image-url)

**Fig. 3.** Nuclear run-on analysis of c-fos transcription. SSV-NRK cells were serum-starved and serum stimulated as described in text. RA (10^{-5} M) was added to the culture 6.5 h before serum stimulation and the cells were maintained in 20% FBS for 30 min until harvesting. The nuclear run-on experiments were performed as described in “Materials and Methods.” DNA blotted on the membrane was for detecting: Slot 1, SS RNA; Slot 2, the sense transcript of the c-fos gene exon 1; Slot 3, the sense transcript of exon 1; Slot 4, the sense transcript of exons 3 and 4; and Slot 5, the sense transcript of exons 3 and 4. +RA, with RA treatment; -RA, without RA treatment.

Cells, meaning that RA does not affect the rate of elongation.

Effect of Cycloheximide. Cycloheximide, an inhibitor of protein synthesis, has been shown to increase the level of c-fos mRNA in quiescent 3T3 cells and also their serum-induced level (13). Termin superinduction, this phenomenon has been explained on the basis of the inhibition by the drug of the synthesis of a labile nuclease that degrades mRNA or of the contact between the mRNA and a ribosome-associated nuclease (24). We tested the effect of RA on the cycloheximide-superinduced c-fos mRNA levels. SSV-NRK cells were starved and serum stimulated as before. Cycloheximide, 50 μg/ml, was added 3 h prior to serum stimulation. As shown in Fig. 4, cycloheximide treatment resulted in a 3-fold increase in the c-fos mRNA level (Lane 3). RA blocked c-fos induction by serum in the presence of cycloheximide (Lane 4), consistent with the notion that RA acts at the level of transcription initiation, whereas the effect of cycloheximide is at the level of mRNA turnover.

Does RA Regulate c-fos mRNA Level by Directly Interfering with a Membrane Signal Transduction Mechanism? The mechanism of action of RA at the molecular level is not fully understood. Earlier models for retinoid action proposed the involvement of both calcium-cyclic AMP and phosphatidyl inositol membrane signal transduction pathways (26-28). The induction of c-fos in fibroblasts following the stimulation by growth factor is very rapid, usually within 5 min, reaching a steady-state level in about 30 min (12, 13). To test whether RA interferes with the signal transduction pathway at the membrane level, we examined the response of c-fos expression to short-term RA treatment. SSV-NRK cells were grown in 10% FBS and starved in 0.5% FBS for 2 days. RA was added to the serum-starved cultures to a final concentration of 10^{-5} M 5 min before addition of 20% FBS. This would ensure that RA could take effect before the phosphatidyl inositol cascade would be activated by serum (in a matter of seconds) (12). The cells were then incubated for an additional 25 min before harvesting. The results demonstrated that a 30-min exposure to RA was insufficient to cause a decrease in c-fos mRNA levels in SSV-NRK cells (Fig. 5). A most likely explanation for this observation is that RA, a lipid-soluble substance, does not directly interfere with the initial phase of signal transduction pathway.

![Graph showing expression of c-fos mRNA](image-url)

**Fig. 4.** Cycloheximide does not block the RA effect on c-fos induction. The SSV-NRK cells were starved and serum stimulated as before. RA and cycloheximide were added 6 and 3 h before serum stimulation, respectively; 2.2 kilobases (kb) indicates the size of c-fos mRNA. CH, cycloheximide.
Fig. 5. Lack of effect on c-fos expression in cells treated with RA for only 30 min. The SSV-NRK cells were starved and serum stimulated as before. RA, \( 10^{-8} \) M, was added 5 min before serum stimulation. Cells were harvested 25 min after the addition of 20% fetal bovine serum containing RA for RNA extraction. Lane 1, with RA, and Lane 2, without RA treatment; 2.2 kilobases (kbs) indicates the c-fos mRNA band. The same blot was stripped and rehybridized with the \( v \text{als} \) probe.

DISCUSSION

Data presented in this paper demonstrate that RA blocks the induction of c-fos mRNA in the simian sarcoma virus-transformed NRK fibroblast line. The inhibition occurs at the level of transcription initiation, as shown in the actinomycin experiments. The results of the cycloheximide experiments are consistent with this interpretation and further suggest that a labile protein factor is not required for the RA action. Our data also show that the RA effects are unlikely to be due to interference of the membrane events. Because c-fos induction is a critical event in transition of G0-G1 to S in quiescent fibroblasts upon serum stimulation (11), the inhibition of c-fos expression may account for its inhibition of the reentry of the serum-starved fibroblasts into cell division cycle.

C-fos is a nuclear oncogene, encoding a product which forms a transcription factor AP-1 with c-Jun (29, 30). AP-1 is known to mediate activation of transcription by serum. The c-fos gene promoter itself contains an AP-1-like sequence (31), suggesting that the gene is positively autoregulated. Other cis-acting elements of the gene include the adjoining SRE that binds to SRF (32, 33). The C-Fos protein, in addition, represses (negatively autoregulates) the c-fos promoter by interacting with the SRF, by an as yet uncharacterized mechanism (34, 35). This opposing effect of C-Fos protein appears paradoxical, but it may serve as a safeguard against excessive accumulation of c-fos mRNA in the cells following serum stimulation (34). A possible site of RA inhibition of c-fos transcription is a cis-acting element within or near SRE. Alternatively, RA may not directly contact DNA, but rather interacts indirectly with the c-fos regulatory elements through other protein factors.

RAR \( \alpha \), \( \beta \), and \( \gamma \) belong to the steroid/thyroid hormone/retinoic acid receptor superfamily (36, 37). The receptors, upon binding to the ligands, recognize specific sequences that are localized upstream from a target gene, resulting in activation (or repression) of that gene. A RAR-binding sequence has been identified as a direct repeat of the motif GTTCAC (38). A simple explanation of our results is that the RAR binds to the retinoic acid response element near SRE. In support of this notion, Schütte et al. reported that AP-1 and RAR recognize a common response element in the human osteocalcin gene (39). However, a search for the RAR-binding sequence within approximately 280 base pairs' 3' of SRE in the murine c-fos gene (available in GENBANK) has been unsuccessful. It is more likely then that RAR interacts with SRF (or other transcription factors) that regulate c-fos expression. This possibility was raised by recent reports that negative regulation of genes by the glucocorticoid hormone could occur via protein-protein interaction between c-Jun and the hormone receptor (40, 41).

In the HL-60 human promyelocytic cell line differentiating into granulocytes, c-fos expression has been shown to be up-regulated by RA (42), and this up-regulation appears to play a role in their differentiation. c-fos up-regulation is also reported for differentiating F9 murine teratocarcinoma cells (43). In a human embryonal carcinoma cell line, NT2/D1, RA has minimal effects on c-fos expression (44). To our knowledge, ours is the first case where c-fos expression is down-regulated by RA in a cell line. The finding may be important in explaining some of the antitumor effects of RA.

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