Serum deprivation induces SV40 early promoter activity

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Abstract. Proliferation and the expression of proliferation-associated genes are modulated by changing the serum concentration in the media of cultured cells. To determine if activity of the SV40 early promoter is modulated by serum, we examined the expression of SV40 early promoter driven marker genes in murine BALB/c 3T3T cells following serum deprivation or serum stimulation. SV40-promoter-regulated β -galactosidase and chloramphenicol acetyl transferase genes were studied following either transient or stable transfection. The results show that serum deprivation of growing cells induces SV40 promoter activity while serum stimulation of quiescent G₀ cells suppresses it. Kinetic analyses show a significant induction of the SV40 promoter activity during the first 2 days of serum deprivation which is maintained at a high level for 15 days. The induction of reporter gene expression by serum deprivation was selective for the SV40 early promoter because such an effect was not observed using the Rous sarcoma viral promoter. Nuclear run-off assays further show that the transcription controlled by the SV40 early promoter is ~twofold greater in cells rendered quiescent by serum deprivation for 72 h than in growing cells cultured in medium containing serum. These results suggest that one reason SV40 T transformed cells commonly fail to undergo quiescence following serum deprivation is that the SV40 promoter is induced.

Modulation of the concentration of serum in cell culture media is a method that has been widely used to study the regulatory mechanisms that control cell proliferation. It has been reported that more than 80 genes can be induced without *de novo* protein synthesis when quiescent G₀ cells are stimulated to grow with serum (Schneider *et al.* 1991). Some of these genes, such as *fos*, *jun* and *myc* proto-oncogenes, are known to induce cell proliferation. Conversely, recent studies have demonstrated several genes which are preferentially expressed in cells rendered quiescent by serum deprivation (Schneider *et al.* 1988, Gustincich & Schneider 1993, Zhan *et al.* 1994). Among them is the *gas-1* gene which has been shown to inhibit cell proliferation (Del Sal *et al.* 1992). These observations have led to the concept that cell growth is controlled by a balance between positive and negative regulatory mechanisms, which are modulated by the microenvironmental signals such as the concentration of growth factors. Therefore, studies concerning the transcriptional regulatory mechanisms of such genes should lead to a better understanding of these important mechanisms.

The viral SV40 early promoter has been extensively studied for the purpose of understanding transcriptional regulation in general. The SV40 early promoter contains a

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GC-rich promoter and an enhancer. The GC-rich promoter contains SP1 binding sites, a TATA box-like domain, and large T antigen binding sites. The enhancer harbours at least 15 transcriptional regulatory cis-elements including the binding sites for AP-1 and NF κ B. Fos and Jun proteins comprise the AP1 complex which is upregulated within minutes after quiescent normal cells are stimulated to grow by serum (Nishikura & Murray 1987, Nakabeppu *et al.* 1988, Kovary & Brovo 1991); but many human cancers show aberrant expression of these and related genes (Almendral *et al.* 1988). The NF κ B transcription factor is also present in virtually all types of cells (Lenardo & Baltimore 1989). Recently, it has been reported that NF κ B is activated during the process of G₀ to G₁ transition following serum stimulation (Baldwin *et al.* 1991) and that NF κ B regulates *c-myc* expression. In this regard, the *c-myc* promoter contains two functional DNA domains for NF κ B binding and the *c-myc* promoter can be highly induced by NF κ B trans-activation (Duyao *et al.* 1990).

Recently, a '14A' DNA domain in the *gas-1* gene promoter which is specifically modulated in serum-deprived quiescent cells as determined by footprinting analysis has been described (de Martin *et al.* 1993). Whether this domain actually upregulates *gas-1* gene transcription following serum deprivation remains to be elucidated. By searching the SV40 early promoter sequences, we have found a homology to this DNA domain (5'-ATAAATAAAAAAAA-3') which is located in the SV40 GC-rich promoter between the T antigen binding sites II and III (Rio & Tjian 1983). This observation suggests that the SV40 early promoter is likely to be sensitive to the microenvironmental signals for cell growth control. To examine this possibility, we have assayed the effects of serum on SV40 early transcription.

METHODS AND MATERIALS

Plasmids, DNA transfection and cell lines

Plasmids pRSVneo (ATCC, No. 37198) and pRSVcat (ATCC, No. 37152) which contain Rous sarcoma viral LTR linked to the coding sequences for neomycin resistance and chloramphenicol acetyltransferase (CAT), respectively, were obtained from American Type Cell Culture Collection. Plasmid pSV₂neo (ATCC, No. 37149), which contains a neomycin resistant gene driven by SV40 early promoter, was also obtained from American Type Cell Culture Collection, whereas plasmid pSV- β -galactosidase, that contains β -galactosidase coding sequences driven by SV40 early promoter, was purchased from Promega (Madison, WI). DNA transfection and selection of stably transfected cells in medium containing neomycin analog G418 were performed according to previously described methods (Tzen *et al.* 1990).

3T3T murine mesenchymal stem cells were originally derived from BALB/c A313T3 cells (Diamond *et al.* 1977). Stably transfected 3T3T subclones include TS839, H1297, TS126 and TS129. TS839 cell line is a subclone derived from 3T3T cells that was stably cotransfected with pRSVcat and pRSVneo followed by 500 μ g/ml G418 selection. H1297 cell line was derived from G418 selected 3T3T cells that were cotransfected with pSV- β -galactosidase and pSV₂ neo. Both TS126 and TS129 cells, which contain integrated CAT sequences driven by SV40 early promoter, have been developed and characterized previously (Tzen & Scott 1993). In selected studies, cell transiently transfected with pSV- β -galactosidase were also used.

Cell culture

Stock cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO) containing 10% bovine serum (HyClone, Logan, UT). Serum deprivation-

induced quiescence was achieved by culture of cells in DMEM containing 0.5% bovine serum for 1 to 4 days. Restimulation of growth of serum deprived cells was achieved by culture in DMEM containing 10% bovine serum. In transiently transfected cultures, serum deprivation was begun within 20 h after transfection and serum deprivation was maintained for 72 h before serum restimulation was induced for a subsequent 18-h interval. Determination of the growth state of cultured cells was performed by the use of standard ^3H -thymidine incorporation assays to measure DNA synthesis.

Enzyme assays

Both CAT and β -galactosidase assays were performed using equal amounts of cellular protein for each sample. CAT assays were performed and quantitated according to the methods described previously (Tzen & Scott 1993). The β -galactosidase assays were performed using commercially available kits (Promega, Madison, WI).

RESULTS

Serum stimulation suppresses the expression of transiently transfected SV40-promoted gene in serum-deprived quiescent cells

BALB/c 3T3T cells were employed to examine if the SV40 early promoter activity is regulated by serum. The 3T3T cell line was derived from BALB/c 3T3 cells which represent the most frequently used cell line to study the interaction of serum growth factors with their receptors that results in the control of cell proliferation.

These cells were transiently transfected with pSV- β -galactosidase, a plasmid containing a β -galactosidase reporter gene driven by an SV40 early promoter. Soon after transfection, the cells were made quiescent by serum-deprivation for 3 days. Then, the quiescent cells were stimulated with medium containing 10% bovine serum; quiescent cells without serum stimulation were used for comparison. Results of two independent assays showed that SV40-promoted β -galactosidase activity was reduced by an average of 45% after serum stimulation of quiescent cells (Table 1).

These results suggested that serum stimulation induced SV40-promoted gene expression, although it is possible that the effect might be associated with transient transfection procedure because it is known that non-integrated plasmid genes are unstable in mammalian cells and that they may be made more unstable by changing the serum concentration in the

Table 1. The effect of serum stimulated on β -galactosidase expression driven by SV40 early promoter in quiescent 3T3T cells harbouring transiently transfected plasmid, pSV- β -galactosidase

Experiment	β -galactosidase activity*		
	Quiescence§	Serum stimulated¶	Serum-induced inhibition (%)
1	0.415	0.245	59
2	0.440	0.138	31

*The β -galactosidase activity was measured spectrophotometrically at OD₄₂₀ after incubating the mixture of cell extracts and substrate, *o*-nitrophenyl- β -D-galactopyranoside.

§Quiescent cells were prepared by culture in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% serum for 3 days.

¶Serum restimulation was performed by adding 10% serum-containing DMEM to quiescent cells.

culture media. Therefore, subsequent studies were performed on stably transfected cell lines.

Induction of stably transfected SV40-promoted gene expression by serum deprivation

Experiments were next performed using stably transfected cells to examine whether serum-deprivation effects SV40-promoted β -galactosidase activity. For this study, stably transfected cells, H1297, were employed. They were established by cotransfection of pSV- β -galactosidase with pSV₂neo, a plasmid expressing neomycin resistance, followed by selection in 500 μ g/ml G418 according to a previously described method (Tzen *et al.* 1990). Cell extracts of both the rapidly growing H1297 cells and quiescent H1297 cells were assayed. Results showed that the β -galactosidase activity of H1297 cells in 10% bovine serum was 0.296, whereas when cells were cultured in 0.5% bovine serum for 24 h, 48 h and 72 h, the activity was 0.440, 0.474 and 0.480, respectively. This result shows that serum deprivation induces SV40-promoted β -galactosidase activity within 24 h and that induction persisted for at least 3 days.

To rule out the possibility of a post-transcriptional effect specific to the β -galactosidase activity, experiments were repeated on cell lines containing the stably integrated chloramphenicol acetyltransferase (CAT) reporter driven by SV40 early promoter. Two murine mesenchymal stem cell lines, i.e. TS126 and TS129, harbouring the SV40-promoted CAT plasmid pSV₂cat were used; they have been characterized previously (Tzen & Scott 1993). TS126 cells contain multiple integrations of pSV₂cat as shown by Southern blotting and thus express relatively high basal levels of CAT activity, whereas TS129 cells show one integration site for pSV₂cat and express low basal levels of CAT activity. Both cell lines were subjected to the serum deprivation as described. The results show that serum deprivation markedly induced SV40-promoted CAT activity in both TS126 and TS129 cells within 3 days (Figure 1). Additional longer term kinetic studies on TS129 cells showed >25-fold induction of

Figure 1. Induction of SV40-promoted CAT activity by serum deprivation. TS126 and TS129 cells are stably transfected cell lines containing SV40-promoted CAT gene. Both TS126 (lanes 1–4) and TS129 (lanes 5–8) were incubated in medium containing 0.5% serum for 24 h (lanes 2 and 6), 48 h (lanes 3 and 7), or 72 h (lanes 4 and 8) to assay the effect of serum deprivation. Cells which were cultured in medium containing 10% serum were exponentially proliferating and served as controls (lanes 1 and 5). The CAT activities were 27% (lane 1), 59% (lane 2), 65% (lane 3), 63% (lane 4), 0.4% (lane 5), 8.9% (lane 6), 7.5% (lane 7) and 6.8% (lane 8).

pSV₂cat in the first 2 days of serum deprivation, 18-fold induction on day 3 (similar to Figure 1), and 12- to 15-fold induction between days 10 and 15 (data not shown).

These results establish that serum deprivation can increase SV40-promoted expression of two reporter genes. From this perspective, a decrease of SV40-promoted gene expression following serum stimulated in quiescent cells appears to reflect a return to the pre-serum deprivation level with respect to SV40 promoter activity.

Specificity of the regulation of SV40 early promoter by serum activity

To evaluate the selectivity of the serum effect on transcriptional regulatory sequences, the SV40 early promoter was replaced by the Rous Sarcoma Viral (RSV) promoter to drive a CAT reporter gene. A stable transfected cell line, designated TS839, was established for this purpose. Both TS126 and TS839 were then subjected to serum deprivation. The results show that CAT activity was induced by serum deprivation of TS126 cells but not of TS839 cells (Figure 2). These data suggest that serum deprivation selectively induces the SV40 promoter.

Transcriptional regulation of SV40 early promoter activity

The induction of gene expression by serum deprivation therefore might be mediated at the transcriptional level because this effect can be abolished by changing the transcriptional regulatory sequences but not by changing the reporter genes. To directly test this possibility, nuclear run-on transcription assays were performed according to the previously described

Figure 2. Specificity of SV40 early promoter to serum deprivation. Both TS126 and TS839 were cultured in the medium containing either 10% serum or 0.5% serum. The CAT activity of the TS126 cells was 8.2% in the presence of 10% serum (lane 1) and increased to 26.3% following serum deprivation after three days (lane 2), whereas the CAT activity of the TS839 cells was 5.3% in the presence of 10% serum (lane 3) and was not induced by serum deprivation for 1 day (6.2%, lane 4), 2 days (7.3%, lane 5), or 3 days (7.8%, lane 6).

procedure (Greenberg & Ziff 1984). In brief, nuclei were harvested from growing TS126 cells cultured in serum or from serum-deprived quiescent TS126 cells. The newly transcribed RNAs were labelled by ^{32}P -UTP and hybridized to the nitrocellulose filter containing excessive amount of denatured plasmid DNA harbouring either CAT or GAPDH coding sequences. The results presented in Figure 3 show that the nascent CAT transcripts, but not control GAPDH transcripts, from serum-deprived cells are ~twofold higher than those from cells cultured in 10% serum-containing medium. These results suggest that serum-deprivation activates the SV40 early promoter at least in part by transcriptional mechanisms.

DISCUSSION

The SV40 early promoter harbours many transcriptional regulatory cis-elements. Among them are binding sites for AP-1 and NF κ B factors. Since AP-1 and NF κ B are generally thought to be serum-responsive transcriptional activation factors (Almendral *et al.* 1988; Baldwin *et al.* 1991), their presence in SV40 early promoter implies that serum treatment should induce SV40 early promoter activity. The current data show instead that serum deprivation and not serum stimulation induces SV40 early promoter activity. This conclusion is supported by the observation that the induction of SV40 early promoter activity by serum-deprivation can be abolished by changing the promoter from the SV40 early promoter to the RSV-LTR, but not by changing the reporter gene from CAT to β -galactosidase. It was also shown that the method of transfection, i.e. either transient or stable, did not change the results. Nuclear run-on transcription assays further show that the transcription of the SV40 early promoter is higher in cells rendered quiescent by serum deprivation than in cells growing in the medium containing serum.

The current data therefore suggest the existence of a serum-deprivation responsive element(s) in the SV40 early promoter. Since it is not known if the '14A' DNA domain is

Figure 3. Run-on transcription assay. Radiolabelled nascent RNA transcripts of serum-deprived quiescent TS126 cells (column A) or exponentially proliferating TS126 cells (column B) were hybridized to a filter containing 50 μg of denatured, linearized plasmid DNA harbouring either CAT coding sequences (row 1) or GAPDH coding sequences (row 2). This slot blot shows the following counts: the CAT transcripts in serum-deprived cells was 59647 cpm (A1); the CAT transcripts in rapidly growing cells was 32764 cpm (B1); the GAPDH transcripts in serum-deprived cells was 32608 cpm (A2); and the GAPDH transcripts in rapidly growing cells was 37079 cpm (B2).

functionally involved in upregulating the transcription of *gas-1* gene upon serum deprivation, we do not know if the '14A' homology in the SV40 early promoter is responsible for our current observation; it is possible that a novel uncharacterized DNA domain is involved. Alternatively, a known cis-element that is regulated by a transcriptional factor complex which is modified by serum deprivation may be involved. The latter could reflect the recent findings of Pfarr *et al.*, who showed that the composition of AP-1 complex can be modulated upon serum deprivation so that the level of JunD increases in the quiescent cells and decreases upon serum stimulation relative to JunB and JunC. In such a system, AP-1 containing mostly JunD would function as a transcriptional repressor whereas AP-1 containing mostly JunB and JunC would function as an activator (Pfarr *et al.* 1994). This latter possibility is supported by a recent observation (Wang *et al.* 1996).

Transcriptional regulators utilized by SV40 early promoter are cellular factors that must be regulated by serum deprivation to control expression of the host's own genes. Therefore, the SV40 early promoter can serve as a target to study the transcriptional regulatory mechanisms of cell quiescence. In this regard, these observations also provide an explanation concerning why SV40 T transformed cells commonly fail to efficiently undergo quiescence following serum deprivation (Estervig *et al.* 1990).

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