Serum independence of transcription from the promoter of an avian retrovirus in *v-src*-transformed cells is a primary, intracellular effect of increased tyrosine phosphorylation

(Rous sarcoma virus/orthovanadate/long terminal repeat/temperature-sensitive oncogene)

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ABSTRACT We found that transcription from the promoter in the long terminal repeat of Rous sarcoma virus in rat 3Y1 cells is dependent on the presence of serum in the culture. However, this serum dependence of transcription was relieved when 3Y1 cells were transformed by the oncogene *v-src*. Crossfeeding experiments showed no evidence for the production of a serum-substituting extracellular growth factor by the transformed cells. Using 3Y1 cells transformed with temperature-sensitive Rous sarcoma virus, we showed that the tyrosine kinase activity of pp60^{v-src} was responsible for the serum-sparing effect on the level of RNA expressed from the viral promoter. Sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases that nonspecifically elevates the level of phosphotyrosine-containing proteins in cells, stimulated transcription from the viral promoter. The effects of both pp60^{v-src} and orthovanadate were resistant to cycloheximide. These results suggest that the serum independence of transcription from the viral promoter in *v-src*-transformed cells was probably due to the constitutive activation of intracellular growth-factor pathways by the tyrosine kinase activity of pp60^{v-src}.

Transformation of a fibroblast by the oncogene *v-src* produces many changes in the physiology of the cell (1). The tyrosine kinase activity and membrane localization of the *v-src* gene product are essential for generating the transformed phenotype (2). However, the mechanism by which *v-src* produces the transformed phenotype is still not clearly understood. In an attempt to elucidate such a mechanism, we decided to focus on one of the salient features of a transformed cell: serum independence.

Our strategy was to select a model promoter that is dependent on serum in the untransformed cell and study how the *v-src*-encoded protein substitutes for serum in the transformed cell. The model promoter was the long terminal repeat (LTR) of Rous sarcoma virus (RSV), the avian retrovirus that carries *v-src*. We found that in the untransformed rat fibroblast cell line 3Y1, serum stimulated transcription from the RSV LTR without requiring new protein synthesis and that half the effect was mediated by the induction of a nuclear CCAAT-binding factor that bound to the LTR (24). We also found that the tyrosine kinase activity of the *v-src* protein increased the amount of the same nuclear CCAAT factor, thereby suggesting a mechanism by which pp60^{v-src} could confer serum independence upon expression from the RSV LTR, and possibly from several other promoters that contain CCAAT elements.

In this paper we show that the transformation of 3Y1 by *v-src* relieved the serum dependence of transcription from the RSV LTR. We demonstrate that the effect was not due to the secretion of extracellular growth factors that substituted for

FIG. 1. RSV LTR in plasmids pRSVcat and pRSVneo. Structure of the RSV LTR at the 3' end of the provirus is shown. The *Pvu* II–*Bst*NI fragment is contained in pRSVcat and pRSVneo. Start site of transcription is indicated as +1. Positions of the TATA box, the enhancer (enh), and the U3, R, and U5 regions are indicated. CCAAT elements referred to in the text are in the antisense strand at positions –65 and –129 relative to the transcription start site. RI, *Eco*RI.

serum, and was dependent on the tyrosine kinase effect of pp60^{v-src}. Nonspecific elevation of the level of phosphotyrosine in the cells by sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases, increased transcription from the LTR. Further, the effects of pp60^{v-src} and vanadate on the RSV LTR could be seen in the presence of an inhibitor of protein synthesis. These results suggest that the serum independence of transcription from the RSV LTR in transformed cells is due to the constitutive activation of intracellular signal-transduction pathways by the tyrosine kinase activity of pp60^{v-src}.

MATERIALS AND METHODS

Plasmids. The plasmids pRSVcat and pRSVneo contain the 3' end of the RSV provirus, including the intergenic region between *v-src* and the 3' LTR, and the whole of the LTR (Fig. 1), cloned upstream from either the bacterial chloramphenicol acetyltransferase (CAT) gene or the neomycin phosphotransferase (NEO) gene (3). Transcripts initiate at the authentic RSV transcription start site, code for CAT or NEO, and are polyadenylated at a simian virus 40 polyadenylation site.

Cells and Viruses. 3Y1 is an immortalized line of cells derived from rat fibroblasts. NY68-3Y1 is a line of 3Y1 transformed by tsNY68, a strain of RSV carrying a temperature-sensitive mutation of *v-src* (4). Chicken embryo fibroblasts (CEFs) transformed by tsNY68 were superinfected with a transformation-defective variant of Schmidt–Ruppel subgroup D (this subgroup can infect mammalian cells). The medium from these doubly infected cultures contained tsNY68 virus pseudotyped as subgroup D and was used to infect 3Y1 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). The cells were main-

tained at 34°C and foci of transformed cells were seen in 10 days. These were isolated by using cloning cylinders, grown up, tested for a temperature-sensitive phenotype by growing them at 39°C, and recloned by dilution. Most of the experiments described in this paper were done with one line, NY68-3Y1.

The SR-3Y1 cell line was derived from 3Y1 by transformation with wild-type Schmidt-Ruppin RSV (5). Experiments were done with a clone derived from a colony in soft agar that maintained its transformed phenotype consistently in DMEM containing 5% calf serum (CS).

3Y1 or NY68-3Y1 cells were transfected with 5 μ g of pRSVneo linearized at a unique *Bam*HI site that is outside the NEO transcription unit, and G418-resistant colonies were selected and pooled to prepare 3Y1-NEO and NY68-3Y1-NEO, respectively.

Western Blotting. NY68-3Y1 cells were maintained in 10% FCS/DMEM at 39°C for 48 hr. After the medium was changed, the cells were shifted down to 34°C and harvested at the indicated times. The procedure and the anti-phosphotyrosine antibody used have been described (6).

Northern Blotting, S1 Nuclease Analysis, Transfections, CAT Assays, Nuclear Run-On Assays. The procedures have been described (24).

RESULTS

Serum Independence of Gene Expression from the RSV LTR in *v-src*-Transformed Cells Is an Intracellular Effect. Transient transfection of pRSVcat into 3Y1 cells showed that the CAT activity per plate was 5-fold greater in the presence of serum than in its absence (Fig. 2A; data quantitated in ref. 24). In contrast, the CAT activity in 3Y1 transformed by *v-src* (SR-3Y1) was not significantly different in the presence or absence of serum, the level of activity in either case being the same as in serum-treated 3Y1. Thus the RSV LTR was suitable for use as a model promoter in that CAT expression directed by it was serum-dependent in 3Y1 and serum-independent in SR-3Y1. The stimulatory effect of serum was seen even on density-arrested, stationary 3Y1 cells, suggesting that cell proliferation was not required (Fig. 2A). The higher CAT activity obtained from the stationary cultures of 3Y1 was due to higher efficiency of transfection in these cultures.

If the serum independence of CAT synthesis in SR-3Y1 were due to the production of extracellular growth factors by the transformed cells, the SR-3Y1 cells would be expected to cross-feed cocultured 3Y1 cells and confer serum independence upon expression of CAT in 3Y1. We found that coculturing 3Y1 with SR-3Y1 did not confer serum independence to the expression from pRSVcat in 3Y1 cells (Fig. 2B). In the reciprocal experiment, SR-3Y1 cells retained their serum independence even upon coculturing with 3Y1 cells. Thus, production of extracellular factors was unlikely to be a major mechanism for the serum independence of pRSVcat expression in SR-3Y1.

Temperature-Sensitive *v-src*-Transformed 3Y1 Line. NY68-3Y1 cells demonstrated morphological transformation upon shift-down from 39°C to 34°C (Fig. 3A). A Western blot of protein lysates from NY68-3Y1 probed with anti-phosphotyrosine antibody showed that there was much more phosphotyrosine-containing protein at 34°C than at 39°C, though not as much as in the wild-type *v-src*-transformed SR-3Y1 cells (Fig. 3B). Upon shift-down to 34°C there was a progressive increase in phosphotyrosine-containing protein. When the shift-down was carried out in serum-free DMEM in the presence of cycloheximide there was still a discernible increase in phosphotyrosine-containing protein (compare lanes 11 and 12). This is expected because the shift-down allows the preexisting *v-src* protein to partially regain its activity and

FIG. 2. (A) Serum dependence of expression of pRSVcat in 3Y1 cells and serum independence of SR-3Y1 cells. 3Y1 stationary: 3Y1 cells were plated at 5×10^5 per 60-mm dish and allowed to grow for 90 hr before transfection of pRSVcat. In the remaining cases, 3×10^5 cells were plated per 60-mm dish 16 hr before transfection. Each plate of cells was transfected with 5 μ g of pRSVcat and maintained for 36 hr in serum-free DMEM (–) or 5% CS/DMEM (+) before being harvested and used for CAT assays. One plate equivalent of lysate was used in each reaction. Thus the CAT activities shown are normalized in each pair (– or + serum) to equal numbers of cells present at the time of transfection before the initiation of serum deprivation. (B) Serum independence of expression of pRSVcat in SR-3Y1 cells is not due to the production of extracellular factors. Transfected: cells transfected with pRSVcat. Six hours after transfection, the cells were trypsinized, pooled, and counted, and 3×10^5 cells were mixed with equal numbers of the indicated cells and plated in 5% CS/DMEM. Twelve hours later, cultures were washed with Tris-buffered saline and maintained in either serum-free DMEM (–) or 5% CS/DMEM (+) for 30 hr before they were harvested. One plate equivalent of lysate was used in each CAT assay shown.

thereby increase the phosphotyrosine content in the cells without any newly synthesized *v-src* protein.

Tyrosine Kinase Activity of pp60^{v-src} Stimulates Expression from the RSV LTR Without Requiring New Protein Synthesis. NY68-3Y1 contains full-length viral genomic RNA and the spliced *env* and *src* mRNAs, all of which initiated in the 5' LTR of the RSV provirus. To test whether the tyrosine kinase activity of pp60^{v-src} influenced the level of RNA initiated at the RSV LTR, total RNA was harvested from NY68-3Y1 at various times after shift-down in the presence or absence of serum. Equal quantities of total RNA were analyzed on a Northern blot to study changes in viral RNA levels (Fig. 4). Though only the *v-src* subgenomic mRNA is shown, identical changes were seen with the genomic and *env* mRNAs. The *v-src* mRNA level dropped rapidly in serum-free DMEM at 39°C. In 5% FCS/DMEM at 39°C, the initial increase in the RNA level following change of medium was short-lived, a finding consistent with our observation that in untransformed 3Y1 cells, the stimulation of the RSV LTR lasted for only 5–10 hr after addition of serum (24).

FIG. 3. Kinetics of morphological transformation and increase in phosphotyrosine-containing proteins upon shift-down of NY68-3Y1. (A) Micrographs showing morphology of NY68-3Y1 cells maintained at 39°C for 72 hr or incubated 14 hr or 48 hr after shift-down to 34°C ($\times 175$). (B) Western blot of proteins, probed with anti-phosphotyrosine antibody. Each lane contained 100 μ g of total protein. The protein lysates were from 3Y1 (lane 1), SR-3Y1 (lane 2), NY68-3Y1 kept at 34°C (lane 3), NY68-3Y1 kept at 39°C for 48 hr (lane 4), and NY68-3Y1 at 1 hr (lane 5), 6 hr (lane 6), 11 hr (lane 7), 24 hr (lane 8), and 48 hr (lane 9) after shift-down from 39°C to 34°C in 10% FCS/DMEM. NY68-3Y1 cells grown at 39°C in 10% FCS/DMEM were transferred to serum-free DMEM with cycloheximide at 50 μ g/ml (lane 10) and kept at 39°C for 8 hr (lane 11) or at 34°C for 8 hr (lane 12) or 18 hr (lane 13). Lane M, molecular size markers, which show protein bands at 200, 97, and 68 kDa.

In contrast, when NY68-3Y1 was shifted to 34°C, the *v-src* mRNA level continued to be high in the absence of serum, suggesting that the active pp60^{v-src} relieved the serum requirement for maintenance of viral RNA levels. Active pp60^{v-src} stimulated the level of viral RNA even in the presence of serum. To rule out the possibility that the temperature changes alone were affecting the level of RNA expressed from the RSV LTR, 3Y1 cells containing pRSVneo integrated in the genome, 3Y1-NEO cells, were maintained at 39°C or 34°C in the absence or presence of serum. The levels of NEO mRNA compared on a Northern blot were found to be the same at the two temperatures (data not shown).

We next tested whether the effect of the tyrosine kinase activity of pp60^{v-src} on the RSV LTR required new protein synthesis. NY68-3Y1-NEO cells growing at 39°C in 10% FCS/DMEM for 72 hr were transferred to serum-free DMEM in the presence or absence of cycloheximide. The cultures were maintained for 8 hr either at the same temperature or after shift-down to 34°C, and the level of NEO transcripts initiating at the LTR was measured by an S1 nuclease assay (Fig. 5). Serum depletion of the cells at 39°C caused a decrease in the amount of NEO transcripts. The "serum-sparing" effect of the active pp60^{v-src} could be seen in a parallel culture shifted down to 34°C (compare lanes 2 and 3). Addition of cycloheximide (10 μ g/ml) to the plates before

shift-down caused a 2-fold increase in the level of NEO transcripts (compare lanes 1 and 4; also seen in 3Y1-NEO, Fig. 6A). However, the serum-sparing effect of pp60^{v-src} was still evident even though protein synthesis was blocked (Fig. 5, compare lanes 5 and 6).

Orthovanadate Stimulates Transcription from the RSV LTR in Serum-Starved Cells. Since the tyrosine kinase activity of pp60^{v-src} induced an increase in RNA expressed from the RSV LTR, we examined whether other stimuli that also increased the phosphotyrosine content of the cells would have a similar effect. This possibility was supported by the finding that high doses of insulin, insulin-like growth factor I, and epidermal growth factor, all of which activate tyrosine kinase receptors, had a serum-sparing effect on the LTR (A.D., unpublished observations). Addition of orthovanadate, an inhibitor of phosphotyrosine phosphatase, to cells has been shown to increase the level of phosphotyrosine-containing proteins and even transform some cell lines (7, 8). Fig. 6 shows the effect of adding sodium orthovanadate to 3Y1-NEO. The level of NEO RNA was determined by using equal quantities of total cellular RNA in a slot-blot analysis and hybridizing with a probe from the NEO gene. A 10-fold increase in the steady-state level of NEO RNA was induced by 150 μ M sodium orthovanadate. Further, part of the stimulation persisted in the presence of cycloheximide. Fig.

FIG. 4. Active pp60^{v-src} relieves the serum dependence of the RSV LTR and increases the levels of viral transcripts. Total RNA from NY68-3Y1 cells under various conditions was hybridized with a nick-translated DNA fragment from a molecular clone of *v-src*. Sections of Northern blots with the *v-src* mRNA are shown. The corresponding ethidium bromide-stained filters are shown below each blot to prove that equal quantities of RNA were present in all lanes (rRNA). The samples at 0 hr contained RNA from NY68-3Y1 kept in 5% FCS/DMEM at 39°C for 72 hr immediately after the medium was changed. (A) Cells maintained at 39°C after the medium was changed to serum-free DMEM (–) or 5% FCS/DMEM (+) were harvested at the times indicated (hr). (B) Same as A except that cells were shifted down to 34°C after medium change to examine the effect of active pp60^{v-src} on the viral RNA level. A shorter exposure of the Northern blot in B is shown to avoid loss of resolution in the 48-hr lane. The 0-hr signals in the two blots are equal.

FIG. 5. Serum-sparing effect of *v-src* on the isolated RSV LTR and in the presence of cycloheximide, shown by S1 nuclease analysis of NEO transcripts in NY68-3Y1-NEO. Ten micrograms of total RNA was used for each lane. The probe was pRSVneo cut and end-labeled at a unique *Nco* I site in the NEO gene. Transcripts correctly initiated in the RSV LTR protect the 950-base-long fragment shown. Cells grown in 10% FCS/DMEM at 39°C for 72 hr were transferred to serum-free DMEM and RNA was harvested immediately (lanes 1 and 4), after 8 hr at 39°C (lanes 2 and 5), or after 8 hr at 34°C (lanes 3 and 6). Lanes 1–3, no cycloheximide; lanes 4–6, cycloheximide (10 μ g/ml) was added at time of medium change.

6B shows that most of the effect of orthovanadate on the NEO RNA could be accounted for by a stimulation of transcription from the RSV LTR as measured by nuclear run-on assay. The effect of orthovanadate on the RSV LTR was not part of a generalized, nonspecific stimulation of transcription, because we found that the promoters for *c-fos*, β -tubulin, and 28S ribosomal RNA genes were not stimulated by the same treatment.

DISCUSSION

The results presented in Fig. 2A suggest that gene expression from the RSV LTR becomes serum-independent upon transformation with *v-src*. Fig. 4 confirms that pp60^{v-src} increases the level of RNA expressed from the RSV LTR in the absence of serum, accounting for the serum independence of expression from pRSVcat in SR-3Y1 cells. We have shown that in untransformed cells at least half the serum responsiveness of the RSV LTR is mediated through a basal transcriptional element, the CCAAT element, which has been described in numerous cellular promoters. Thus, the serum independence of expression of pRSVcat in *v-src*-transformed cells is likely to be reflected in the expression of several cellular genes, and such a widespread effect on multiple promoters may contribute to the serum independence of cell growth seen upon transformation by pp60^{v-src}.

There are many examples where the transformation of a cell by an oncogene confers the ability to grow independent of certain extracellular growth factors (1, 12–16). In some of the instances cited above, the factor-independent cells were found to secrete a protein into the extracellular medium, accounting for the independence from exogenously added factor. For example, *v-mos*-transformed fibroblasts secrete transforming growth factor (TGF-) α and TGF- β (17), and a variety of transformed cells secrete proteins that resemble platelet-derived growth factor (18). Upon transformation by *v-src*, myeloid cells produce a growth factor called cMGF (13) and 3T3L1 murine fibroblasts produce a TGF- β -like factor and a protease-sensitive mitogen (19) that are detectable in the extracellular medium. In other instances, extracellular factors cannot be detected in media conditioned by the transformed cells (20, 21). The cross-feeding experiment (Fig. 2B) showed that the serum independence of RSV LTR in SR-3Y1 was not explained by the production of extracellular growth factors by these cells.

An intracellular mechanism by which pp60^{v-src} induces the RSV LTR could be secondary to the synthesis of new proteins that activate signal-transduction pathways intracellularly. Such a mechanism may involve the production of an intracellular growth factor that stimulates its cognate receptor intracellularly, or even the synthesis of a receptor for extracellular growth factors. Intracellular growth factors have been detected in the human osteosarcoma cell line U-2 OS CL 6 (22) or the *bcr-abl*-transformed myeloid cell line FDC-P1 (21). The alternative possibility for an intracellular

FIG. 6. Orthovanadate mimics the effects of serum and *v-src* on the RSV LTR. (A) Slot blot analysis of NEO transcripts in 3Y1-NEO. After serum deprivation (serum-free DMEM) for 48 hr (0-hr point), the cells were exposed to 150 μ M sodium orthovanadate and total RNA was prepared at the times indicated. The levels of NEO RNA were analyzed by slot blot hybridization of 3 μ g of total RNA per slot with the nick-translated NEO DNA probe described in B below. All signals were quantitated by densitometry of the autoradiograms, normalized to the signal from the 0-hr time point, and plotted (Δ). In a parallel experiment, cycloheximide (50 μ g/ml) was added before vanadate (Δ). (B) Nuclear run-on analysis of 3Y1-NEO. Radiolabeled run-on transcripts were prepared from cells that were in serum-free DMEM for 48 hr (–Vanadate) and from parallel cells that were exposed to 150 μ M sodium orthovanadate for 6 hr before harvest (+Vanadate). The blots contained 1 μ g of gel-purified DNA fragment or 5 μ g of plasmid per slot. The positions of these immobilized probes on the blots are indicated by the key below the blots: NEO, *Hind*III–*Nco* I fragment of pRSVneo containing the NEO coding portion; FOS, plasmid pFos, which has the 1.0-kilobase *Pst* I fragment of murine *v-fos* (9) cloned into pUC18; TUB, pTub, which has the 1.7-kilobase chicken β -tubulin cDNA cloned into the *Pst* I site of pBR322 (10); 28S, p28S, which contains the 4.8-kilobase *Sal* I–*Eco*RI fragment of a murine 28S rRNA genomic DNA clone in pBR322 (11); PUC18, pUC18; ϕ X, bacteriophage ϕ X174 DNA.

mechanism is that the kinase activity of pp60^{v-src} directly activates signal-transduction pathways by posttranslational modification or intracellular transport of preexisting proteins. In view of the stimulation of the RSV LTR by pp60^{v-src} and vanadate in the presence of cycloheximide (Figs. 5 and 6A), we favor the hypothesis that pp60^{v-src} directly activates intracellular signal-transduction pathways.

Is the tyrosine kinase activity of pp60^{v-src} important for the stimulation of expression from the RSV LTR and for the serum-sparing effect? The kinetics of increase of phosphotyrosine in cellular proteins, especially the 60-kDa protein, upon shift-down of NY68-3Y1 cells (Fig. 3B) parallels the increase of viral RNA levels (Fig. 4). High doses of insulin, insulin-like growth factor I, and epidermal growth factor added separately to serum-starved 3Y1 stimulate the expression of pRSVcat in transient-transfection assays, though not as effectively as serum (unpublished observations). Vanadate

also produces a primary induction of the level of RNA expressed from the RSV LTR (Fig. 6A). These three lines of evidence suggest that like the morphological transformation of cells (2), the serum-independence of expression from the RSV LTR is dependent on the tyrosine kinase activity of pp60^{v-src}.

The findings that (i) serum stimulates transcription from the RSV LTR and increases the level of a CCAAT-binding factor (24), (ii) orthovanadate stimulates transcription from the LTR, and (iii) the tyrosine kinase activity of pp60^{v-src} increases the level of RNA directed by the LTR and increases the amount of CCAAT-binding factor suggest that any increase in cellular phosphotyrosine (by vanadate or pp60^{v-src}) increases the amount of CCAAT-binding activity, thereby stimulating transcription from the RSV LTR. This model also explains how pp60^{v-src} substitutes for serum in SR-3Y1 cells.

In view of the primary effects of serum, vanadate, and pp60^{v-src} on the RSV LTR, we suggest that at least part of the increase in CCAAT factor produced by pp60^{v-src} is a primary effect. However, the initial effect on the transcriptional machinery may be amplified if it results in increased production of limiting components of the signal-transduction machinery like pp60^{v-src} or the CCAAT-binding factor. That this could indeed be the case is suggested by the relatively slow increase in the amount of pp60^{v-src} (Fig. 3B) and nuclear CCAAT-binding protein (unpublished observations) present in NY68-3Y1 after shift-down.

The tyrosine kinase activity of pp60^{v-src} may have a similar stimulatory effect on the RSV promoter in CEFs. Orthovanadate increased the level of viral RNA in serum-deprived CEFs infected with td107 (a transformation-defective variant of RSV), in a manner similar to the effect on the RSV promoter in 3Y1 cells (unpublished observations). Further, in tsNY68- or tsNY72-infected cultures of CEFs, when total cellular RNA was harvested at various times after shift-down to the permissive temperature, we found a 3- to 4-fold increase in the level of viral RNAs within 0.5–1 hr after shift-down (A.D. and K. Barker, unpublished observations). The rate of increase in viral RNA corresponded well to the rate of increase in phosphotyrosine-containing proteins after shift-down (6). However, the increase in viral RNA was transient, much like what has been reported for several other v-src-induced messages in CEFs (23).

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