Original Articles

The 3' Untranslated Region of the Chicken c-*src* Protooncogene Modulates Gene Expression

(v-*src* / posttranscriptional regulation / mRNA stability)

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Abstract. Tight regulation of the Src tyrosine kinase activity is essential for a variety of cellular processes, namely transitions of the cell cycle. The peaks of Src activity are dependent on its posttranslational modifications as well as on the regulation of gene expression. The 3'UTRs of mRNAs are often crucial for rapid changes of the protein level. The chicken c-*src* 3'UTR effects on gene expression have been explored. The c-*src* 3'UTR decreased the *in vivo* tumorigenic potential of the *src*-activated mutants in chickens. This corresponds with the finding that the c-*src* 3'UTR reduced the Src protein and src mRNA levels and luciferase activity *in vitro*. Our results suggest that the chicken c-*src* 3'UTR plays a role in the negative control of gene expression, either transcriptionally or posttranscriptionally.

The tyrosine kinase c-Src is involved in several cellular singalling events including activation of tyrosine kinase receptors, G-protein-coupled receptors, integrins, etc. A wide variety of the Src substrates become phosphorylated during distinct phases of the cell cycle, cytoskeleton rearrangement, cell attachment to extracellular matrix, phospholipid signalling and apoptosis. The *src* gene is important in embryonic development and has a tissue-specific pattern of expression. The importance of the tight regulation of Src kinase activity is exemplified by the oncogenic potential of viral dysregulated v-Src mutants. Human c-Src is found to be overexpressed and/or activated in a broad scale of human tumours, notably those of the colon, breast, lung, pancreas and liver (reviewed in Irby and Yeatman, 2000). Moreover, Src may play a role in tumour progression and in the development of the metastatic phenotype. An activated truncated mutant of the human *SRC* gene has been found in 12% of highly advanced human colon cancers and was suggested to promote metastasis (Irby et al., 1999).

The precise regulation of *src* expression is required for G1/S (Broome et al., 1996; Erpel et al., 1996) and G2/M (Roche et al., 1995) transitions during the cell cycle. Peaks of Src activity are achieved through transient protein posttranstranslational modifications as well as through regulation of its expression. It is well known that activation of chicken c-Src depends on phosphorylation of tyrosine 416 and dephosphorylation of tyrosine 527. In addition, phosphorylation of some Ser and Thr residues is found at mitosis. The transcriptional regulation is exerted by two promoters described for the human c-*SRC* gene: the housekeeping promoter SRC1A (Ritchie et al., 2000) and the 1.0 kilobase upstream promoter SRC1α, which displays a tissue-restricted pattern of expression with highest levels present in stomach, kidney, and pancreas (Bonham et al., 2000). Furthermore, the posttranscriptional regulation is frequently used to ensure the rapid changes in protein expression, e.g. in the case of c-*fos*, c-*jun* and c-*myc* protooncogenes. The effects of the mRNA untranslated regions (UTRs), especially the 3'UTRs, on the mRNA turnover often play a critical role in the tight regulation of proteins with short half-lives. The regulation of *src* mRNA stability has already been reported by Dehm et al. (2001), who observed that human colon cancer cell

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Abbreviations: ARE – AU-rich element, AUF-1 – ARE-binding/degradation factor, CEF – chicken embryonic fibroblast, CMV – cytomegalovirus, hnRNP D – heterogeneous nuclear ribonuclear protein D, IgG – immunoglobulin class G, LTR – long terminal repeat, MOPS – 3-[N-morpholino]propanesulphonic acid, N-CBZ-Leu-Leu-Leu-al – carbobenzoxyl-leucinyl-leucinyl-leucinal, NRC – neuroretinal cell, ORF – open reading frame, PBS – phosphate-buffered saline, p.i. – post-injection, PVDF – polyvinylidene fluoride, SDS – sodium dodecyl sulphate, S.E.M. – standard error of the mean, SV40 – simian virus 40, UTR – untranslated region.

lines with increased c-*src* mRNA and kinase activity had decreased c-*src* mRNA stability.

The 3'UTRs were described to modulate protein production at various stages of mRNA turnover including nuclear export, mRNA degradation, intracellular mRNA localization and efficiency of translational initiation (reviewed in Grzybowska et al., 2001; Pesole et al., 2001). More specifically, these effects reside in shorter *cis* elements with a characteristic structure and/or protein-binding capacity. For example, AU-rich elements (AREs) are suggested to be the key players in controlling mRNA stability (Caput et al., 1986; Shaw et al., 1986). These AREs are bound by RNA-binding proteins including the family of Hu proteins (Levine et al., 1993; Gao et al., 1994) and heterogeneous nuclear ribonuclear proteins D (hnRNP D) also called AUF-1 protein (ARE-binding/degradation factor) (Kiledjian et al., 1997). HuA and hnRNP D are suggested to play opposite roles in the turnover of ARE-containing mRNAs, HuA protein stabilizing (Fan and Steitz, 1998; Peng et al., 1998) and hnRNP D protein destabilizing (Loflin et al., 1999) mRNA carrying AREs in their 3'UTRs. Polyubiquitinylated hnRNP D protein was suggested to direct the degradation of bound mRNA by the RNase activity of the proteasome machinery (Jarrousse et al., 1999; Laroia et al., 1999; Laroia et al., 2002).

The 3'UTR sequence is known in the case of chicken (1839 nucleotides; Yatsula et al., 1994a), quail (1880 nucleotides; Yatsula et al., 1994a), mouse (675 nucleotides; GenBank Accession No. BC018394), human (2488 nucleotides; GenBank Accession No. AF077754), *Xiphophorus xiphidium* (1559 nucleotides; GenBank Accession No. X64658), *Drosophila melanogaster* c-*src* (Dsrc41 gene; 914 nucleotides of the 3'UTR; GenBank Accession No. D42125) and partially in the case of rat c-*src* (the first 372 nucleotides, GenBank Accession No. AF157016). A high sequence similarity can be found between chicken and quail 3'UTRs. The regional sequence similarity of 47 nucleotides in length can be found between chicken or quail and human 3'UTRs. The AREs with the core consensus sequence AUUUA are found in the c-*src* 3'UTR of chicken (one ARE), quail (one ARE), human (one ARE), *Xiphophorus* (one ARE), and *Drosophila* (4 AREs). Although not defined in *src* mRNA, the role of the 3'UTR in the *lck* mRNA turnover was reported. Transient downregulation of the steady-state level of the tyrosine kinase $p56^{lck}$ mRNA during full activation of T lymphocytes was shown to depend on the *lck* 3'UTR (Vanhée-Brossollet and Vaquero, 1997). The human *lck* 3'UTR is 451 nucleotides long (Perlmutter et al., 1988), shows no significant sequence similarity to the chicken c-*src* 3'UTR and contains no ARE.

v-src^{PR2257}, an activated and tumorigenic mutant of chicken c-*src* protooncogene, is transduced by the replication-defective PR2257 retrovirus (Geryk et al., 1989; Yatsula et al., 1996). In comparison to the c-*src*, the v-src^{PR2257} contains a single frameshift mutation, which replaces the negative regulatory tyrosine 527 of c-*src* by valine 527 and extends the original open reading frame (ORF) of c-*src* by 55 amino acids. Importantly, the PR2257 retrovirus transduces the upstream half (0.9 kb) of the c-*src* 3'UTR captured by multiple recombination events during the PR2257 evolution (Yatsula et al., 1994a). This event should most likely have occurred in the course of (-) RNA synthesis by recombination between c-*src* transcripts involving the 3'UTR. In the course of *in vivo* passages of the PR2257 virus, a large deletion of the 3' terminus of the c-*src* 3'UTR was observed concomitantly with a tenfold increase in the transforming titre (Yatsula et al., 1994b). These previous findings led us to the suggestion that the virus eliminates the c-*src* 3'UTR elements playing a negative role in viral expression and replication.

To address the potential role of the chicken c-*src* 3'UTR, we used the activated mutants of the chicken c-*src* gene and the *luciferase* reporter gene to monitor the effects exerted by chicken c-*src* 3'UTR on the expression of homologous and heterologous coding sequences, respectively. We conclude that the chicken c-*src* 3'UTR downregulates gene expression either transcriptionally or posttranscriptionally.

Material and Methods

Plasmids used

The pLTRE/2257 construct (Yatsula et al., 1996) contains the entire PR2257 genome in the pBR322 backbone. The pUSRCUTR construct (Yatsula et al., 1994a) contains the entire sequence of the chicken c-*src* 3'UTR (1.8 kb) in sense orientation in the pUC18 backbone. To withdraw the c-*src* 3'UTR readily, the pTAUTR construct was created by insertion of the entire PCR-amplified c-*src* 3'UTR bounded by the *Xba*I restriction sites of the pCR2.1 vector (Invitrogen, Carlsbad, CA).

The pLSL construct (Fig. 1) contains the LTR, v-src^{PR2257}, LTR cassette in the pBR322 vector backbone and was created as SB construct by Briešťanská and Plachý (1996). To obtain the pLSUL construct (Fig. 1), the *Dra*III-*Asu*II fragment of the pLTRE/2257 was substituted by a *Dra*III-*Acc*I fragment from the pUSRCUTR, thereby joining the entire c-*src* 3'UTR in sense orientation downstream to the LTR-driven v-src^{PR2257}. The LTR sequences in all constructs are derived from the Pr-C RSV.

The pLSFL construct was created as pLTR'[c-*src*F527] by Yatsula et al. (1996). It contains the LTR, c-*src*F527Y, LTR cassette (Fig. 1) with the activated mutant of the chicken c-*src* gene, where the negative regulatory tyrosine 527 is replaced by phenylalanine. pLSFU+L and pLSFU-L constructs were created by insertion of the *Eco*RI fragment of pTAUTR containing the entire c-*src*

3'UTR downstream to the c-*src*F527Y into the *Pfl*MI site of the pLSFL construct either in sense or antisense orientation, respectively (Fig. 1). In the same way, the 1 kb *Xba*I restriction fragment of the pAS2-1 vector (BD Biosciences, Palo Alto, CA) was inserted into the pLSFL construct to obtain the pLSFNSPL construct (Fig. 1).

The set of plasmids pcSF, pcSFU+, pcSFU-, pcSFNSP, pcU+ and pcU- was derived from the pcDNA3 vector (Invitrogen) containing the gene for neomycin resistance. The *Eco*ICRI fragment of the pLSFL contains the entire coding region of the c-*src*F527Y. In the pcSF this *Eco*ICRI fragment was inserted into the pcDNA3 *Eco*RV polycloning site (Fig. 1). The entire c-*src* 3'UTR, either in sense or antisense orientation, was cloned as an *Xba*I fragment downstream to c-*src*F527Y into the pcSF *Xba*I site to obtain the pcSFU+ and pcSFU- constructs, respectively (Fig. 1). In the same way, 1 kb *Xba*I restriction fragment of the pAS2-1 vector (BD Biosciences) was used to obtain the pcSFNSP construct (Fig. 1). The entire c-*src* 3'UTR, either in sense or antisense orientation, was inserted as an *Xba*I fragment into the pcDNA3 *Xba*I polycloning site to obtain pcU+ and pcU- constructs, respectively (Fig.1).

The set of luciferase constructs pLU+, pLU- and pLNSP was derived from the pGL3 vector (Promega, Madison, WI). The c-*src* 3'UTR either in sense or antisense orientation was cloned as an *Xba*I fragment downstream to the *luciferase* into the *Xba*I polycloning site of the pGL3 vector to obtain the pLU+ and pLU- constructs, respectively (Fig. 1). In the same way, the 1 kb *Xba*I fragment of the pAS2-1 vector (BD Biosciences) was used to obtain the pLNSP construct (Fig. 1).

Tumour induction in vivo

Linearized plasmid DNA (0.5, 1.0 or 5.0 µg of oncogenic inserts) was used for subcutaneous inoculation of 3-week-old chickens of the CC.R1 inbred line (Plachý et al., 1989) or endogenous virus-free P line (Chernov et al., 1985). Growth of the primary tumours was monitored as described (Svoboda et al., 1992).

Cell culture, stable transfections and cumulative growth curves

Chicken embryonic fibroblasts (CEFs) prepared from 10-day-old embryos were cultivated at 37°C or 40°C in Dulbecco's modified essential medium supplemented with 5% foetal calf serum (Invitrogen, Carlsbad, CA), 5% newborn calf serum (Invitrogen, Carlsbad, CA) and 1% chicken serum. For stable transfection, 1 x 10^6 CEFs were seeded per 60-mm dish and medium was changed 1 h before transfection. Five µg of expression plasmid were transfected by the standard calcium phosphate procedure (Martin et al., 1986), selection with 200 µg/ml G418 was started 48 h after transfection and maintained for 7–10 days.

Chicken neuroretinal cells (NRCs) freshly dissected from 1.5 of 8-day-old chicken embryos were cultivated in Eagle's basal medium supplemented with 5% foetal calf serum as described by Pessac and Calothy (1974). Five, 10 or 20 µg of expression plasmid DNA were transfected by the calcium phosphate procedure (Martin et al., 1986), selection with 600 µg/ml G418 was started 7 days after transfection and maintained for 13 days. Proliferating NRC colonies were coloured by crystal violet when selection was completed.

The growth rate of CEFs and NRCs was assessed by means of cumulative growth curves. One million of cells per 100 mm dish were seeded and counted at regular intervals.

Western blot analysis

Protein extracts of cells were prepared using RIPA lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with aprotinin (Sigma, St. Louis, MO; final concentration 1.5 µg/ml). Total protein concentration was assessed and normalized by the standard Bradford assay. Extracted proteins were separated on 10% SDS-polyacrylamide vertical gel electrophoresis, transferred onto the PVDF membrane (Amersham Biosciences, Piscataway, NJ), blocked with 4% milk in TBS-T (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% Tween 20) and incubated overnight at 4°C with either mouse EC10 anti-chicken Src monoclonal antibody (Upstate Biotechnology, Charlottesville, VA; final concentration 1 µg/ml) or mouse mAb327 anti-v-Src monoclonal antibody (Ab-1; Oncogene; final concentration 1 µg/ml). After washing four times with TBS-T, the membranes were incubated for 5 h at 4°C with goat anti-mouse IgG antibody conjugated to peroxidase (Amersham; dilution 1 : 1000), followed by four washes with TBS-T. Immunoreactive proteins were detected with the chemiluminescence system ECLplus (Amersham) according to the manufacturer's instructions.

RNA extraction and Northern blot analysis

Total cellular RNA was isolated using either the RNA Now reagent (Biogentex, Seabrook, TX) according to the manufacturer's instructions or the slightly modified RNA isolation method according to Chomczynski and Sacchi (1987). Fifteen µg of total RNA were electrophoresed through a 1% agarose MOPS/formaldehyde gel, capillary blotted to nitrocellulose membrane (Amersham) in 20x SSC, hybridized at 42°C with 0.8 x 10^6 cpm of denatured probe per ml of hybridization buffer (50% formamide, 5x SSC, 1x Denhardt solution, 20 mM phosphate buffer, pH 7.0, 10% dextran sulphate, 0.3% SDS, 100 µg/ml calf thymus DNA) and washed in 0.2x SSC, 0.2% SDS and 0.1x SSC, 0.1% SDS at 50°C. The probe was labelled by random hexa-

nucleotide-primed incorporation of [α-^{32}P]dCTP (Klenow labelling kit, Promega) into the 2093 bp *Nco*I restriction fragment of the pLSFL construct containing the chicken c-*src* ORF according to the manufacturer's instructions.

Luciferase assays

The amount of 1 x 10^6 CEFs per 60-mm dish were seeded and co-transfected with 1.5 μg of reporter luciferase plasmid and 1.5 μg of β-actin-driven β-galactosidase expression plasmid. Cells were harvested 48 hours after transfection in 1x reporter lysis buffer (Promega) and luciferase activity was measured with a CliniLumat (Berthold, Pforzheim, Germany) luminometer using a Luciferase Assay System (Promega). Luciferase activities were normalized according to β-galactosidase activities assessed as $O.D._{420nm}$.

For the luciferase assay in stably transfected cell clones, the transfected and G418-selected CEF clones were harvested from the 60-mm dishes in the PBS buffer, the cells were pelleted, lysed by 3 cycles of dry ice freezing and thawing in 100 μl of 250 mM Tris, pH 7.6. Fifteen μl of cellular lysate were mixed with 200 μl of ATP buffer and 200 μl of luciferin, and luciferase activity was measured using the manual Berthold luminometer. The luciferase activities were normalized according to the protein concentration measured by the standard Bradford's assay.

Results

3'UTR of chicken c-src reduces the in vivo tumorigenic potential of activated c-src mutant

To test the effect of c-*src* 3'UTR on the *in vivo* tumorigenic potential of the v-*src*^{PR2257}, we have used pLSL and pLSUL constructs (Fig. 1) with the c-*src* 3'UTR absent or present, respectively, downstream to the LTR-driven v-*src*^{PR2257}. Chickens of the CC.R1 and P lines were inoculated with oncogenic constructs and tumour growth at the site of inoculation was monitored. The entire cloned PR2257 provirus served as a positive control. In both CC.R1 and P lines, the size of tumours induced by pLSUL was lower than that induced by pLSL on day 35 p.i. (data not shown). This suppressive effect was even more pronounced when CC.R1 chickens were inoculated with a low dose of pLSL and pLSUL oncogenic inserts: in this case, the pLSUL construct failed to induce tumours in contrast to the pLSL construct (Fig. 2a). We conclude that the c-*src* 3'UTR downstream to the v-*src*^{PR2257} reduces the *in vivo* tumorigenic potential of v-*src*^{PR2257}.

To confirm the suppressive effect of the c-*src* 3'UTR on the tumorigenic potential, we have used another c-*src* activated mutant called *c*-srcF527Y, which has previously been shown to be tumorigenic in the chicken CC.R1 line, albeit to a lesser extent than v-*src*^{PR2257} (Yatsula et al., 1996). The pLSFL construct contains the LTR-driven c-*src*F527Y. The pLSFU+L and pLSFU-L constructs contain the c-*src* 3'UTR cloned downstream to the c-*src*F527Y in sense or antisense orientation, respectively (Fig. 1). The pLSFNSPL construct contains the non-specific fragment cloned downstream to the c-*src*F527Y (Fig. 1). The LTR, v-*src*, LTR plasmid (described as pH-19KE in Hejnar et al., 2001) containing the v-*src* from PR-C RSV was used as a positive control. The pLSFL, pLSFU+L, pLSFU-L and pLSFNSPL constructs were used for tumour induction in CC.R1 chickens. The tumorigenic activity of the pLSFL construct was lower than that of the pLSL observed in previous experiments or of the LTR-driven v-*src* used as a positive control. The presence of the c-*src* 3'UTR in sense (pLSFU+L), but not in antisense (pLSFU-L), orientation reduced the tumorigenic activity of the c-*src*F527Y to zero (Fig. 2b). This suggests a relative specificity of the mechanism underlying the tumour growth retardation by the 3'UTR *in vivo*. Unexpectedly, the pLSFNSPL construct containing the non-specific fragment of plasmid DNA was non-tumorigenic (Fig. 2b). In this case, the loss of tumorigenicity was probably of different, immune-based, mechanism (see section Discussion).

c-src 3'UTR potentiates cell cycle re-entry of neuroretina cells initiated by c-srcF527Y but has no effect on cell growth parameters

We employed chicken embryonic cells, either the CEFs or the NRCs. The NRCs are quiescent in culture unless they are induced to proliferate by expression of a mitogene (Pessac and Calothy, 1974). c-*src*F527Y was shown to induce cell division of normally quiescent NRCs in culture (Yatsula et al., 1996). To test the potential effects of the c-*src* 3'UTR on NRC stimulation by c-*src*F527Y *in vitro*, we have used pcSF and pcSFU+ constructs contrasting in the presence of the c-*src* 3'UTR downstream to cytomegalovirus (CMV) immediate early promoter-driven c-*src*F527Y (Fig. 1). Both constructs are derived from the pcDNA3 vector, so that the transfected cells can be selected using G418. The NRCs stably transfected with pcSF or pcSFU+ constructs started to divide concurrently 8–10 days after transfection. However, the proportion of dividing cell colonies was higher for the pcSFU+ construct than for the pcSF construct. The effect of the pcSFU- construct, which contains the c-*src* 3'UTR in antisense orientation downstream to CMV-driven c-*src*F527Y (Fig. 1), on the NRCs was the same as for the pcSFU+ construct (Fig. 3). It was possible to passage cells transfected with all three constructs and they continued growing for the next 5–7 passages. The rates of *in vitro* growth measured as cumulative growth curves were not significantly different for cells transfected with different constructs (data not shown).

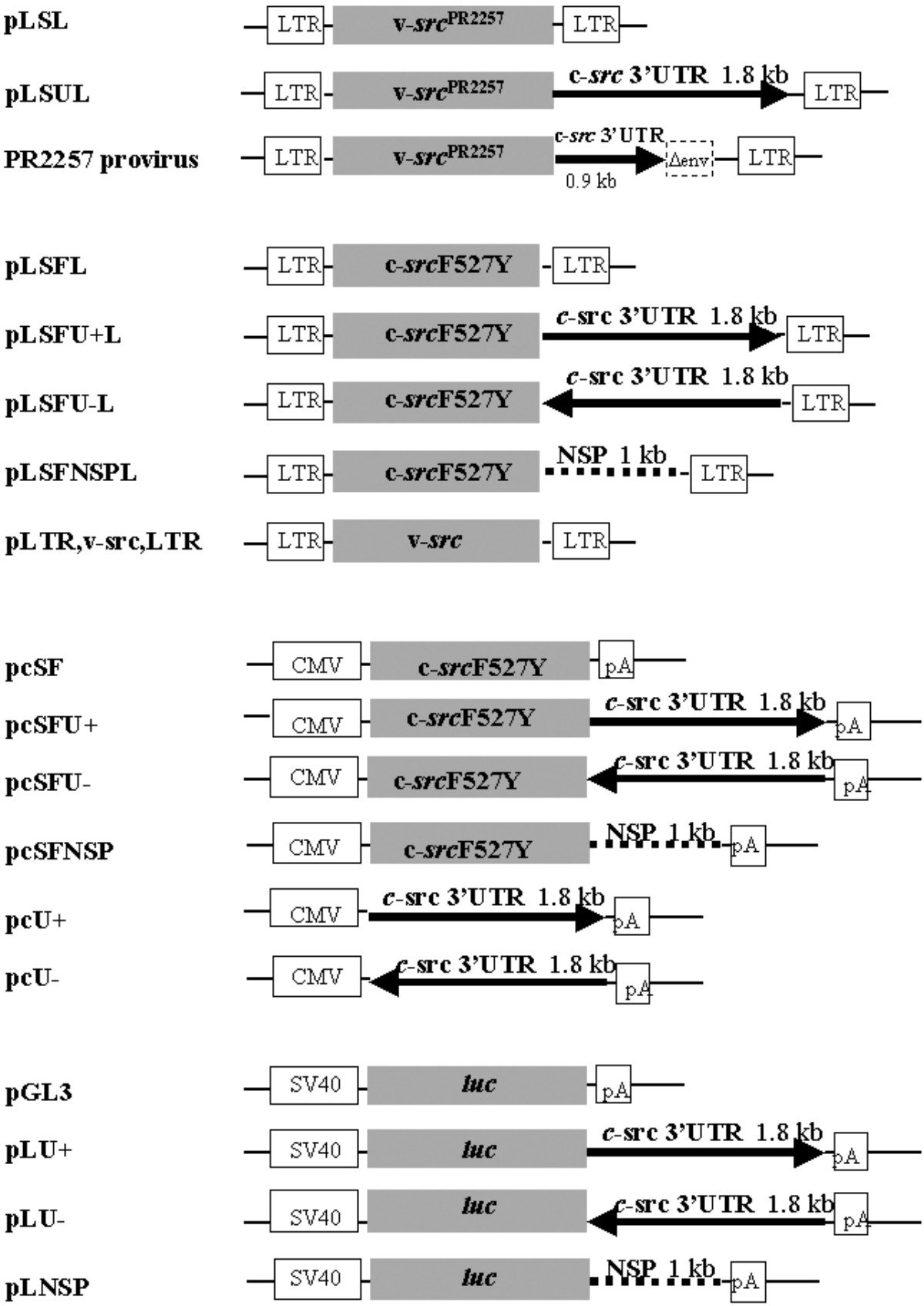


Fig. 1. Schematic figure of constructs used. The c-*src* 3'UTR is either in sense or antisense orientation. LTR represents the long terminal repeat of the Prague strain of Rous sarcoma virus, subgroup C. Δenv represents deleted env of the PR2257. CMV represents the cytomegalovirus immediate early promoter. pA represents the polyadenylation signal. NSP represents the non-specific fragment. SV40 represents the promoter of the SV40 virus.

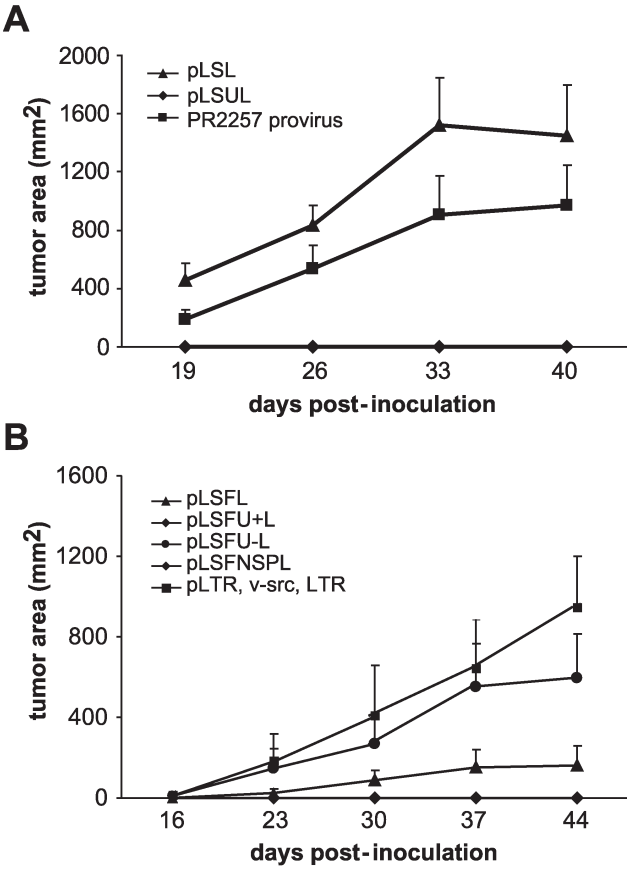


Fig. 2. (a) Kinetics of tumour growth after inoculation of 0.5 μg of pLSL, pLSUL and cloned PR2257 provirus oncogenic inserts into the chickens of the CC.R1 line. The mean + S.E.M. (standard error of the mean) of tumour areas of six birds are presented. (b) Kinetics of tumour growth after inoculation of 5 μg of pLSFL, pLSFU+L, pLSFU-L, pLSFNSPL and pLTR,v-src,LTR oncogenic inserts into the chickens of the CC.R1 line. The mean + S.E.M. of tumour areas of eight birds are presented.

The non-transfected NRCs or cells transfected with the pcDNA3 empty vector remained quiescent. The stable transfection of the pcU+ or pcU- constructs containing the c-*src* 3'UTR in sense or antisense orientation, respectively, downstream to the CMV promoter have not shown any effect on NRC division as compared to the empty pcDNA3 vector (Fig. 3).

In the same way as for the NRCs, we have tested the effect of the c-*src* 3'UTR on the growth rate of adherent CEFs in culture. Similarly, the cumulative growth curves for CEFs stably transfected with pcSF, pcSFU+, pcSFU- and pcSFNSP constructs were not significantly different (data not shown).

Altogether, we conclude that the c-*src* 3'UTR, irrespective of its orientation, facilitates the cell cycle reentry of NRCs initiated by c-*src*F527Y. However, it has no effect on cell growth *in vitro* measured as cumulative growth curves.

c-src 3'UTR reduces luciferase activity when located downstream to luciferase

To confirm the negative c-*src* 3'UTR effect on the expression of an adjacent gene, we performed luciferase activity assays. The entire c-*src* 3'UTR, either in sense or antisense orientation, was cloned downstream to the simian virus 40-driven (SV40) *luciferase* gene (pLU+ and pLU- constructs, respectively; Fig. 1). The parental pGL3 vector alone or with a 1 kb non-specific DNA fragment was used as a negative control. All constructs were co-transfected with a β-galactosidase-expressing plasmid into the CEFs. The luciferase activity was assessed 48 h later and normalized with respect to the β-galactosidase activity. The pLU+ construct reduced the luciferase activity to 20% as compared to the pGL3 or pLNSP constructs (Fig. 4). The pLU- construct reduced the luciferase activity to 5% as compared to the pGL3 or pLNSP constructs. Therefore, the c-*src* 3'UTR reduced expression of the *luciferase* gene as well.

c-src 3'UTR modulates accumulation of the Src protein

The *in vivo* and *in vitro* effects of the c-*src* 3'UTR could be attributed to differences in *src* gene expression. Therefore, we quantified the Src protein by direct Western immunoblots in the chicken cells stably transfected with pcSF, pcSFU+, pcSFU- and pcSFNSP constructs and cultivated at 40°C (Fig. 1). Essentially, the c-*src* 3'UTR in sense (pcSFU+) and antisense (pcSFU) orientations reduced the level of the Src protein in both NRCs and CEFs (Fig. 5). NRCs stably transfected with pcSFNSP contained a reduced level of the Src protein while the CEFs stably transfected with pcSFNSP contained the same level of the Src protein as the cells stably transfected with pcSF.

Taking together with the *in vivo* results (see above), we can conclude that the presence of the c-*src* 3'UTR downstream of the c-*src*F527Y in sense orientation is required for tumour growth retardation in chickens *in vivo*, but both sense and antisense orientation is effective in diminution of the Src protein level in chicken cells *in vitro*.

c-src 3'UTR reduces the accumulation of src mRNA

To assess whether the Src protein level reflects the *src* mRNA level, we analysed the level of *src* mRNA in stably transfected chicken cells by Northern blot analysis. CEFs and NRCs were stably transfected with pcSF and pcSFU+ constructs. In the cells transfected with the pcSFU+ construct, the srcF527Y mRNA level was lower than in the pcSF-transfected cells. We obtained these results for both NRCs and CEFs at 40°C as well as at 37°C (Fig. 6). Therefore, the c-*src* 3'UTR reduces the srcF527Y mRNA level either by transcriptional downregulation or by specific mRNA decay.

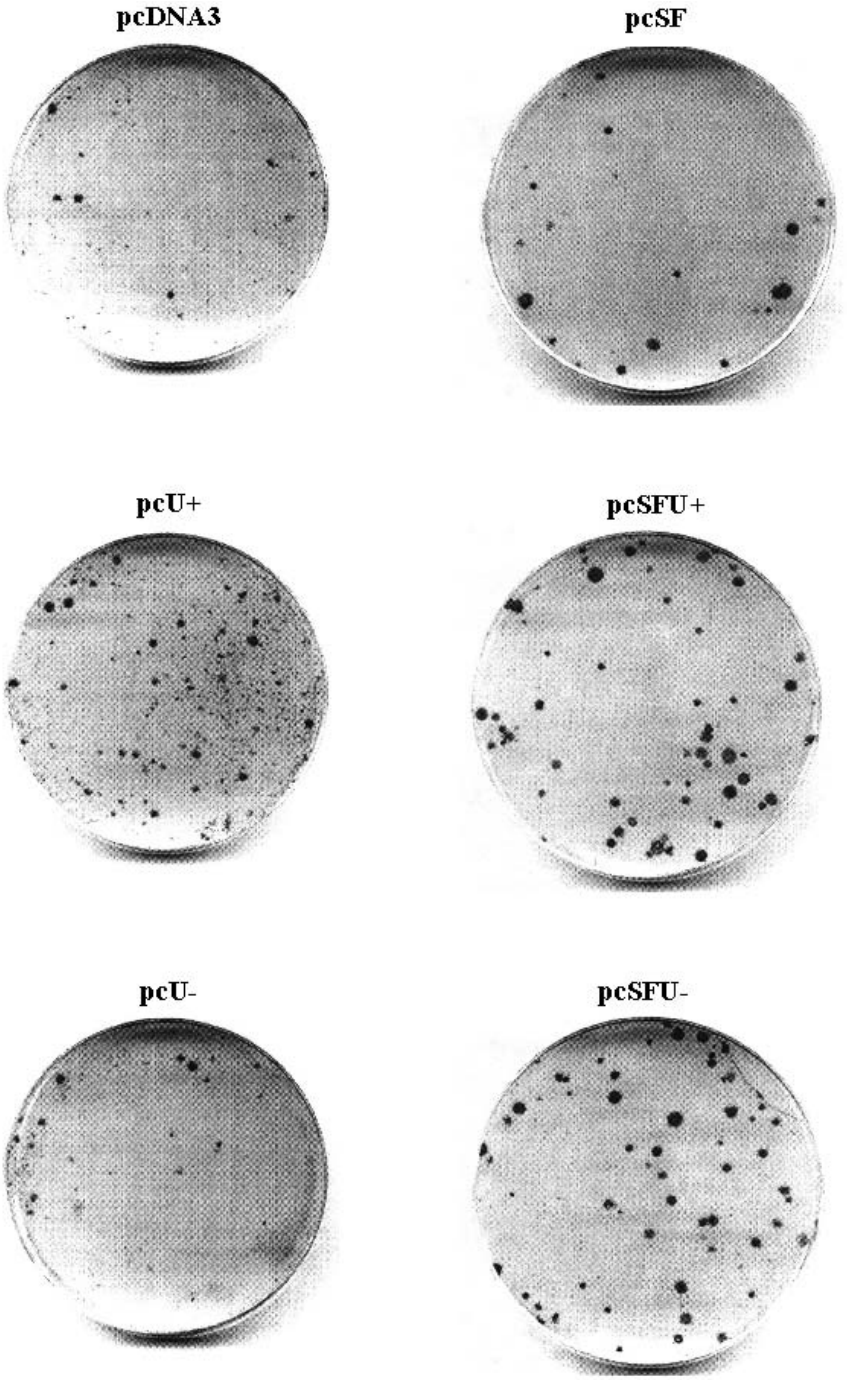


Fig. 3. Effect of the c-*src* 3'UTR on the NRC cell cycle re-entry initiated by the c-SrcF527Y. NRCs were stably transfected with 5 μg of pcDNA3, pcSF, pcSFU+, pcSFU-, pcU+ and pcU- constructs as indicated in Material and Methods and colonies growing after the G418 selection were visualized by crystal violet. Only the NRCs transfected with pcSF, pcSFU+ and pcSFU- constructs continued growing.

Discussion

In the present work, we have shown that the c-*src* 3'UTR plays a role in the negative control of gene expression irrespective of the promoter and gene used, in either *in vivo* or *in vitro* assays. Our indirect findings support the suggestion that this c-*src* 3'UTR effect could rather act at the posttranscriptional level, which includes mRNA stability or translational initiation.

The results obtained from the luciferase transient reporter assays (see section 3.3) form the crucial evidence of our findings that, irrespective of its orientation *in vitro*, the c-*src* 3'UTR reduces gene expression (Fig. 4). Also, in the CEFs stably transfected with either the *luciferase* gene or the *luciferase* gene with the c-*src* 3'UTR downstream, the luciferase activity was reduced in the presence of the c-*src* 3'UTR (data not shown).

Consistently with its control of *luciferase* expression and irrespective of its orientation, the c-*src* 3'UTR in *cis* reduced the accumulation of the Src protein in both types of cells, CEFs and NRCs, at 40°C (Fig. 5; see section *c-src 3'UTR modulates accumulation of the Src protein*). However, the c-*src* 3'UTR effect was exerted, and in NRCs even the non-specific fragment reduced the Src protein level, contrary to the non-specific fragment in CEFs, which had no effect on Src expression. In agreement with reduced Src protein accumulation, we found less *src* mRNA in the CEFs and NRCs stably transfected with pcSFU+ (the c-*src* 3'UTR present) than in the cells stably transfected with pcSF (Fig. 6; see section *c-src 3'UTR reduces the accumulation of src mRNA*).

The c-*src* 3'UTR potentiates c-*src*F527Y-mediated cell cycle re-entry of quiescent NRCs (Fig. 3), without changing the growth rate of either, the NRCs or normally dividing CEFs (see section *c-src 3'UTR potentiates cell cycle re-entry of neuroretina cells initiated by c-srcF527Y but has no effect on cell growth parameters*). The precise mechanism of this phenomenon and its relation to the above-mentioned effects of the c-*src* 3'UTR are not known. One possible explanation is based on the hypothesis that high levels of the Src kinase are harmful to cells. In fact, activated forms of the Src protein were shown to be less stable than either wild-type or kinase-inactive

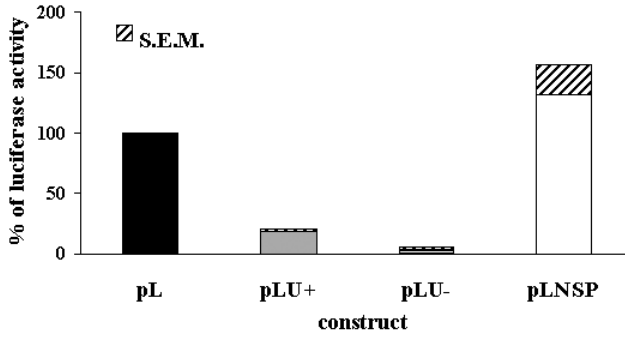


Fig. 4. Normalized luciferase activity of the pGL3, pLU+, pLU- and pLNSP constructs in transient assays (CEFs). Luciferase activity of the pGL3 empty vector was taken as 100%. Mean activities + S.E.M. of five separate experiments are presented.

Src mutants and were found to be polyubiquitinated and degraded by the proteasome (Hakak and Martin, 1999; Harris et al., 1999). The NRCs require SrcF527Y protein expression to re-enter the cell cycle and, at the same time, the levels of the SrcF527Y protein kinase in the proliferating NRC cell populations is below the harmful treshhold. The c-*src* 3'UTR-dependent lower accumulation of the Src protein in NRCs transfected with the pcSFU+ results in more surviving and proliferating colonies. On the contrary, less surviving NRC colonies transfected with the pcSF are selected due to the higher level of Src kinase expression. Similarly to the NRCs, we suggest that the elevated level and activity of the Src protein tyrosine kinase gave a growth disadvantage to the population of transfected CEFs that expressed high levels of Src protein, and this leads to the selection of "resistant" CEFs at the initial phases after transfection. Therefore, the potential c-*src* 3'UTR effects on the Src protein level could be combined with other cell regulatory mechanisms.

To further ascertain the effect of the c-*src* 3'UTR on the growth of tumours induced by activated *src* genes *in vivo*, we used chickens previously characterized for the response to challenge with DNA constructs harbouring *src* genes (Svoboda et al., 1992; Plachý et al., 1994). We found that the c-*src* 3'UTR unambiguously reduced the *in vivo* oncogenic potential of activated mutants of the *src* gene (Fig. 2; see section *3'UTR of chicken c-src reduces the in vivo tumorigenic potential of activated c-src mutant*). Contrary to the bulk of *in vitro* assays, which are independent of the c-*src* 3'UTR orientation, only the sense orientation is effective for tumour growth suppression in chickens. No tumour suppression with the c-*src* 3'UTR in antisense orientation suggests a specific *in vivo* regulatory mechanism not required *in vitro*, and can serve as adequate control for *in vivo* experiments. On the other hand, the non-specific fragment (intended control sequence) cloned downstream of the c-*src*F527Y had a tumour-suppressive effect. The "non-specificity" of the bacterial sequence used can be, however, questioned, because of the presence of the short immunostimulatory consensus sequences containing unmethylated CpG dinucleotides. These bacterial sequences are known to induce a potent antigen-specific cytotoxic lymphocyte response (Tokunaga et al., 1984; Cho et al., 2000). To confirm the c-*src* 3'UTR effects on the transforming capacity of the c-*src*F527Y *in vitro*, we have used the colony formation assay in semi-solid agar, which is one of the used *in vitro* characteristics of oncogenically transformed cells. The stably transfected pcSFU+ construct caused 10 times lower CEF colony formation than the pcSF construct (data not shown). The number of colonies formed by pcSFU+-transfected CEFs was only slightly higher than the background of pcDNA3 empty vector-transfected CEFs.

The negative effect exerted by the c-*src* 3'UTR on gene expression can be exerted either at the transcriptional or at the posttranscriptional level. As a typical transcriptional silencer acts both upstream and downstream, we were interested whether the c-*src* 3'UTR placed upstream to the *luciferase* gene reduces the luciferase activity in the same manner as downstream. We have cloned the c-*src* 3'UTR in sense orientation upstream to the *luciferase* in the pGL3 vector (pU+L construct). Surprisingly, the luciferase activity of pU+L was six times higher than the luciferase activity of the pGL3 vector (data not shown). The measured activities of pGL3 and pNSPL, where the 1 kb non-specific fragment is cloned upstream to the *luciferase*, were equal. As the 3'UTR does not behave as a typical transcriptional silencer, it could be suggested that the c-*src* 3'UTR reduces the mRNA level posttranscriptionally.

A 3'UTR can target mRNA posttranscriptionally to degradation by the ribonuclease activity of the proteasome via bound polyubiquitinylated hnRNP D proteins (Jarrousse et al., 1999; Laroia et al., 1999; Laroia et al., 2002). Therefore, we applied the proteasome inhibitor N-CBZ-Leu-Leu-Leu-al (MG132; Sigma) on cultivated CEFs stably transfected with a construct containing the *luciferase* gene or the *luciferase* gene with the c-*src* 3'UTR cloned downstream. Comparison of the proteasome inhibitor effect on the luciferase activity was, however, strongly affected by the MG132-mediated inhibition of luciferase activity, confirmed recently by Deroo and Archer (2002), for both luciferase and β-galactosidase reporters. When applied on CEFs stably transfected either with the pcSF or pcSFU+, the Src protein level displayed no significant changes or raise during the 26 h of MG132 treatment, respectively (Fig. 7). Regarding the differential effect of the MG132, these results suggest that the effect of the c-*src* 3'UTR could also include the mRNA degradation in the proteasome.

Another potential effect of the c-*src* 3'UTR can be exerted at the level of mRNA/protein localization. We followed the Src protein distribution in the stably trans-

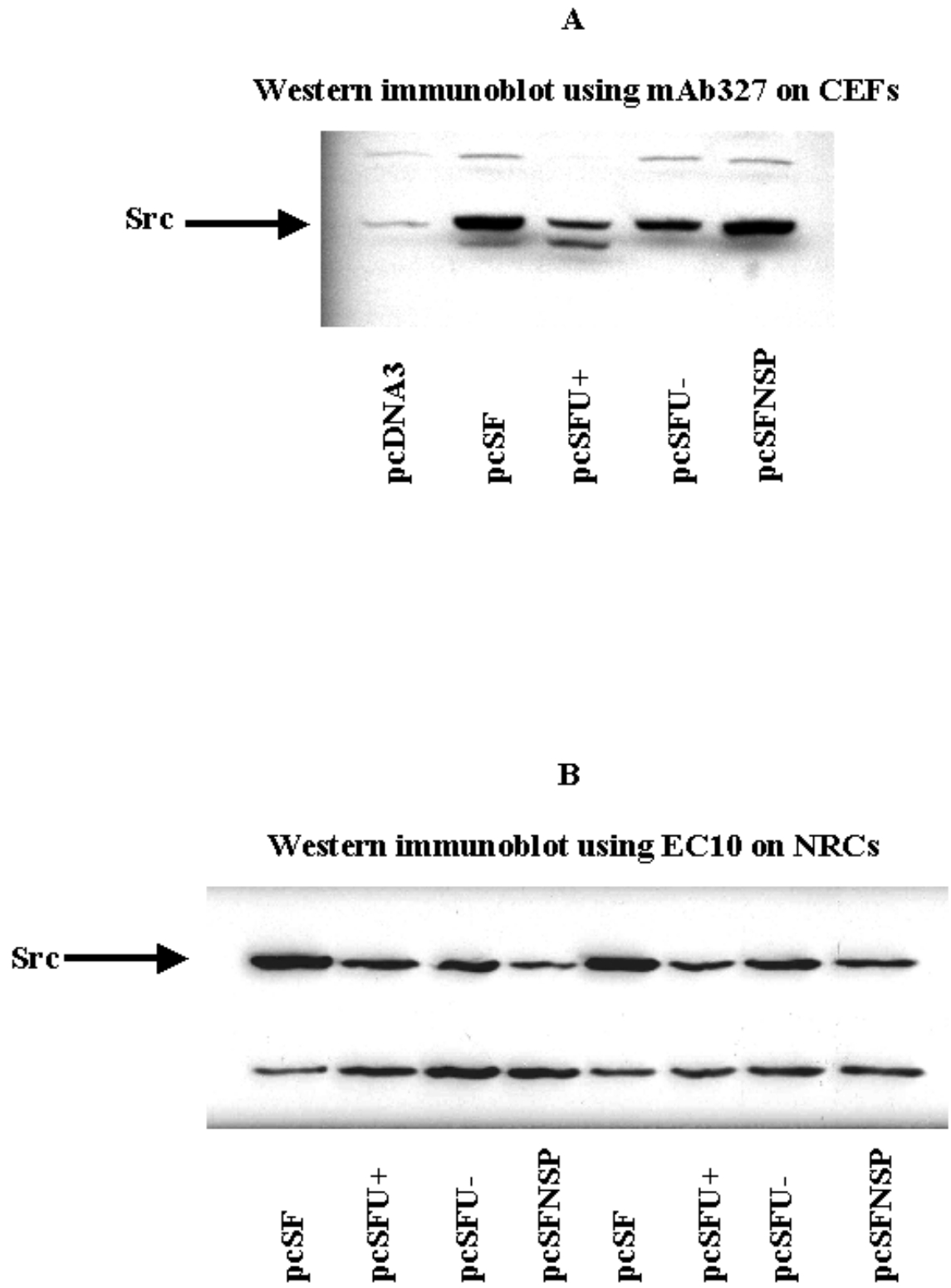


Fig. 5. Western immunoblot analysis of the c-*src* 3'UTR effect on gene expression. (a) CEFs were stably transfected as indicated in Material and Methods and cultivated at 40°C. Loaded normalized cellular protein lysates were incubated with mouse anti-v-Src monoclonal antibody (Ab-1 or mAb327; Oncogene; final concentration 1 μg/ml) and immunoreactive proteins were detected with the chemiluminescence system ECLplus (Amersham). Representative immunoblot of four separate experiments is depicted. The pcDNA3 line represents mock control CEFs. (b) NRCs were stably transfected as indicated in Material and Methods and cultivated at 40°C. Loaded normalized cellular protein lysates were incubated with mouse EC10 anti-chicken Src monoclonal antibody (Upstate Biotechnology; final concentration 1 μg/ml) and immunoreactive proteins were detected with the chemiluminescence system ECLplus (Amersham). Two independent representative immunoblots of four separate experiments are depicted.

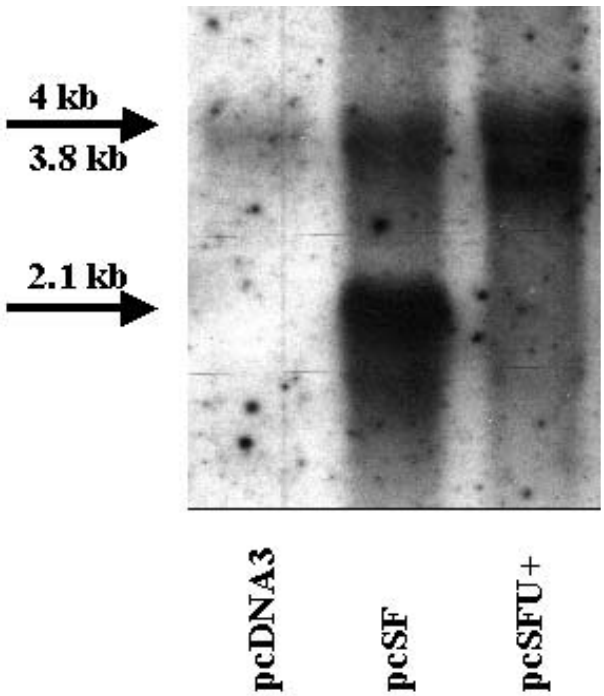
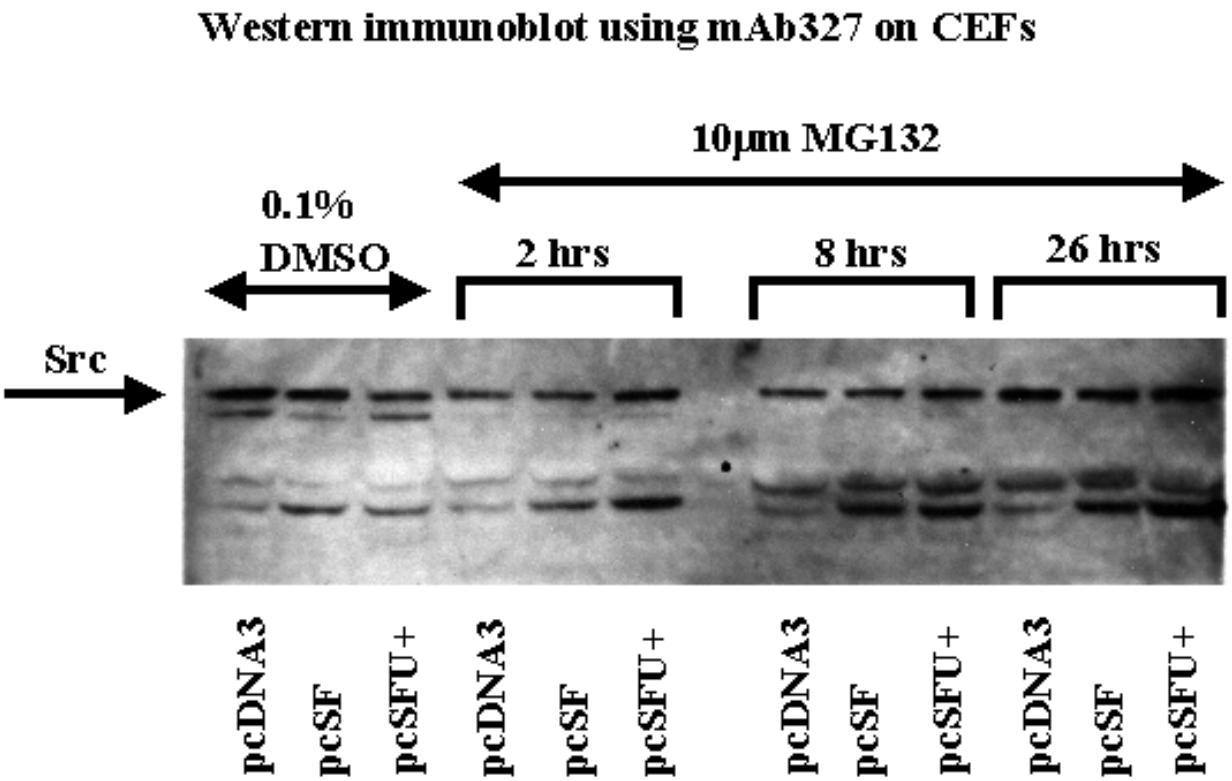


Fig. 6. Northern blot analysis of the *src* mRNA accumulation. CEFs were stably transfected as indicated in Material and Methods and cultivated at 37°C. Northern blot with 15 μg of total RNA loaded in each line was hybridized to the pLSFL *Nco*I fragment (c-*src*F527Y ORF) of 2093 b. Representative blot of three separate experiments is depicted. The pcDNA3 line represents mock control CEFs. About 4 kb is the size of the endogenous c-*src*+c-*src* 3'UTR mRNA and 3.8 kb is the size of the exogenous c-*src*F527Y+c-*src* 3'UTR mRNA. 2.1 kb is the size of the exogenous c-*src*F527Y mRNA.

fected CEFs and NRCs using immunofluorescence. However, we observed no difference in the Src protein localization between the cells stably transfected with pcSF and pcSFU+ (data not shown).

The low sequence similarity between chicken and human c-*src* 3'UTRs concerns the region of 47 nucleotides between positions 1792 and 1837 of chicken c-*src* 3'UTR and positions 1990 and 2036 of human c-*src* 3'UTR. The AREs are conserved between chicken and quail c-*src* 3'UTRs (beginning at nucleotide 1654 and 1671, respectively). However, the ARE existing in human c-*src* 3'UTR is not included in the short similarity region with chicken and quail 3'UTRs. Searching for sequence similarity with the chicken c-*src* 3'UTR in the 3'UTR databases brought no positive results, not excluding the existence of the short *cis* motives or the secondary structures with a regulatory function.

Fig. 7. Western immunoblot analysis of the proteasome inhibitor MG132 effect on c-*src* 3'UTR-dependent Src protein accumulation. CEFs were stably transfected as indicated in Material and Methods and MG132 was added (final concentration 10 μM in 0.1% DMSO) for 2, 8 or 26 h. 0.1% DMSO added to the medium served as a negative control. Loaded normalized cellular protein lysates were incubated with anti-*v*-Src monoclonal antibody (Ab-1 or mAb327; Oncogene; final concentration 1 μg/ml) and immunoreactive proteins were detected with the chemiluminescence system ECLplus (Amersham). Representative immunoblot of two separate experiments is depicted. The pcDNA3 line represents mock control CEFs.



The *in vivo* and *in vitro* results can be easily reconciled in several points. Namely, the presence of the c-*src* 3'UTR reduced expression of either the *src* or *luciferase* gene independetly of the promoter used, thus resulting in a lower tumorigenic capacity of the mentioned oncogenes.

Despite the above-mentioned corresponding results, some apparent discrepancies still remain to be explained, concerning the specificity of the c-*src* 3'UTR effect and its molecular mechanism. Further experiments are necessary to complete the picture of the c-*src* 3'UTR regulatory mechanism.

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