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Studies on the Activity of the Long Terminal Repeat of Rous Sarcoma Virus in Animal Cells and in Yeast

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STUDIES ON THE ACTIVITY OF THE LONG TERMINAL REPEAT OF ROUS SARCOMA VIRUS IN ANIMAL CELLS AND IN YEAST.

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ACKNOWLEDGEMENT.

This being the last part of the thesis to be actually written, it is immensely gratifying to finally get down to the business of acknowledging all the kindness showered on me over the past six years. Surprisingly, learning my way into how "science" gets done, coincided with a gradual discovery of how I want to live the rest of my life, and the two learning processes have become indelibly interlinked: one would not have been possible without the other.

Keeping this in mind, it is no surprise that my thanks go to friends that I made in the lab, particularly, Sally Kornbluth, Bruce Mayer, Mark Stoeckle, Kathy Barker, Sumio Sugano, Michi Hamaguchi, Fred Cross and Thambi Dorai, and several of the respective "spouse-persons". Perhaps the greatest degree of gratitude goes to Saburo Hanafusa for running such a wonderful lab where it is possible to learn about work *and* life and enjoy the process along the way. I hope some of the magic has rubbed off on me to help re-create that charming atmosphere wherever I go.

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ABSTRACT.

Transcription from the Rous sarcoma virus (RSV) Long terminal repeat (LTR) in untransformed rat 3Y1 fibroblasts is dependent on the presence of serum. Within an hour of addition of serum to a serum-deprived culture there is a 5 fold stimulation in the level of transcripts initiated at the LTR. This stimulation does not require synthesis of new proteins. Mutations in the RSV LTR revealed that serum-stimulated transcription was mostly dependent on two CCAAT boxes in the LTR, though other upstream sequences may play a secondary role. Serum caused the rapid appearance of a nuclear protein that binds to the two CCAAT boxes. This serum-induced CCAAT factor was also bound by CCAAT sequences from other promoters, e.g. those of human heat shock protein 70, human c-Ha-ras, human histone 1 etc, but not by the adenovirus origin of replication, or the SV40 enhancer core sequence. This data suggests that the serum induced CCAAT factor is related to CP1 or CP2 rather than the NF1 or C/EBP types of CCAAT binding factors. The abundance of the factor in the nucleus is increased by serum even in the presence of inhibitors of new protein synthesis.

The serum dependence of transcription from the RSV LTR is lost in v-*src* transformed 3Y1, and cross-feeding experiments showed that the mechanism did not involve the production of extra-cellular growth factors. Temperature-sensitive (ts) v-*src* transformed 3Y1 was used to demonstrate that the tyrosine kinase activity of v-*src* can (a) substitute for the serum-requirement of the RSV LTR, (b) increase the level of transcripts initiated in the LTR even in the presence of serum, (c) exert its serum-sparing effect on the RSV LTR in the absence of new protein synthesis, and (d) increase the amount of CCAAT binding factor in the nucleus. Orthovanadate, an inhibitor of tyrosine-phosphatases, which non-specifically elevates the level of phosphotyrosine in the cells, can mimic the effects of serum and v-*src* on transcription from the RSV LTR.

The RSV LTR directed accurate initiation of transcripts in Saccharomyces cerevisiae, about 60 bases downstream from the TATA box that is used in animal cells. Mutations in the LTR revealed that the authentic TATA box was absolutely necessary for the transcription, and the CCAAT boxes that are responsive to serum in animal cells, were acting as "upstream activating sequences". The absence of the TATA box or the CCAAT boxes gave a phenotype to the yeast carrying the mutant derivatives of RSVCAT, making it possible to establish a genetic system for cloning genes for yeast and mammalian transcription factors, and do structure-function analysis on them. The activity of the RSV LTR in yeast is not stimulated by lactate, and is not decreased by mutations in the HAP2 or HAP3 genes, suggesting that the "UAS" activity of the CCAAT boxes is mediated by yeast CCAAT binding factors other than HAP2/HAP3. While a high level of v-*src* is toxic to the yeast, expression of very low levels of the oncogene may be stimulating the CAT activity from the RSV LTR about two fold. If this can be seen at the phenotypic level, it may be possible to establish a genetic system to study how v-*src* influences gene expression in yeast.

CHAPTER-1: INTRODUCTION.

Two approaches to studying transformation of cells by v-*src*.

Transformation of cells in culture by Rous sarcoma virus has been extensively studied as a model for understanding the mechanism of carcinogenesis (Hanafusa, 1977). This has given us tremendous insight into how normal cellular genes can be picked up by retroviruses and be converted into viral oncogenes. It turns out that the mechanism of this conversion itself gives a clue as to how the oncogene transforms cells. In the case of c-*src*, transduction into a retrovirus itself involved truncation of a C-terminal regulatory region (Lerner et al., 1984; Dutta et al., 1985), followed by multiple mutations in the rest of the gene. All of these changes presumably increase the tyrosine kinase activity of the oncogene and result in the transforming phenotype (reviewed in Jove and Hanafusa, 1988; Wang and Hanafusa, 1988).

The minimal requirement for transformation has been studied extensively by in vitro mutagenesis of both v- and c- *src*. Transformation requires (a) an elevated tyrosine kinase activity achieved by the mutations referred to above and by the linkage of the *src* gene to a strong promoter (Jacobovits et al., 1984) and (b) a membrane anchoring myristylation signal that directs the v-*src* protein to the plasma membrane presumably to phosphorylate critical substrates in that subcellular fraction (Cross et al., 1984; Pellman et al., 1985a, 1985b). However, though mutagenesis of v-*src* is very effective in defining domains of the protein required for transformation it can only lead to some educated guesses as to how those domains contribute to the phenotype.

Since the tyrosine kinase activity is absolutely essential for transformation, one approach stemming from the "educated guesses" has been to catalogue the phosphotyrosine containing proteins in the transformed cell. Defining the pathway to the

transformed phenotype from v-*src* by this approach, however, is currently stymied by the large number of cellular proteins that are phosphorylated on tyrosine in v-*src* transformed cells (Hamaguchi et al., 1988).

The second approach is to select a particular phenotype seen in the transformed cell, define it in molecular terms and work backwards from there to the tyrosine kinase activity of v-*src*. Such a strategy has indeed been taken in studying the decreased adhesiveness of transformed cells. Studies by several groups have led to elucidation of the diminished secretion of fibronectin and the decreased affinity of the fibronectin receptor for fibronectin in the transformed cell (Plantefaber and Hynes, 1989; and references therein).

Looking around for a thesis project, I opted for the latter approach. I wanted to define one parameter of the transformed cell in molecular terms and then try to work backwards from there to v-*src*.

Why does an avian retrovirus pick up a cellular oncogene ?

Over the decades many retroviruses have been studied, and it is rather peculiar that whenever a cellular gene is transduced into a retrovirus, it always appear to be an oncogene. One possible reason is selection imposed by the observer, given that most of the retroviruses under scrutiny used to be studied because of their "cancer inducing" properties. However, the situation changed with the appearance of human retroviruses and yet there do not appear to be naturally occurring retroviruses that have picked up a cellular "non-oncogene".

Another observation that may be of interest, is the frequent loss of a non-transforming, in vitro created mutant of an oncogene from a retrovirus, when it is passaged in culture (Foster D. A., personal communication; Jong S.-M. and L.-H. Wang,

personal communication). Finally, if non-transforming cellular genes can be passaged in a retrovirus, there is the frequent appearance of mutants that have activated the transforming ability of the gene (Iba et al., 1984; Levy et al., 1986; Hanafusa T., personal communication; Wang L.-H., personal communication).

Considering all of the above, it could be hypothesised that the cellular oncogene is transduced into the retrovirus and converted into a transforming viral oncogene because it aids the virus in some way. Temin (1967a) and Humphries and Temin (1972 and 1974) showed that RSV could infect a cell and not produce viral particles unless the cell passes through mitosis at least once. It could therefore be suggested that the virus picks up an oncogene because it forces the cell through mitosis and initiates viral replication. However, it is possible that transformation by the oncogene, v-*src*, influences viral replication not only at the beginning of virus production but in a more continuous manner well after the initial production. In fact, to be of interest as a phenotype in the transformed cell that could be studied in molecular terms and would lead back to v-*src*, it would have to be an effect that is seen well after virus production has started, when the cell has a full-blown transformed phenotype. Is there such an effect of v-*src* on viral replication ?

The question can be rephrased as : if there is such an effect on virus replication, where does one look for it ? There are several steps in the viral replication cycle that take place continuously in the transformed cell. Specifically, these are transcription of genomic and subgenomic viral RNA, translation of viral proteins, assembly of viral particles and finally their release. Of these, the more easily studied steps clearly relate to the transcription, processing and translation of viral genes. Since most of the viral transcription signals are located in about 400 bp of viral sequence called the "long terminal repeats" (LTR), it seemed prudent to address the issue of viral transcription.

We have discovered that transcription from the RSV LTR is dependent on the presence of serum, and that v-*src* relieves this serum-dependence.

Oncogenes and transcription.

Oncogenes usurp normal cellular pathways of growth control. There are oncogenes that resemble peptide growth factors, e.g. v-*sis* is the PDGF B chain (Waterfield et al., 1983; Doolittle et al., 1983), and others that resemble peptide growth factor receptors with tyrosine kinase activity, e.g. v-*erb*B is derived from the EGF receptor (Downward et al., 1984), c-*fms* from the CSF-1 receptor (Sherr et al., 1985). Some oncogenes appear to transduce signals from cell surface receptors to other proteins on the inner side of the plasma membrane, e.g. *ras* (Masters and Bourne, 1986) and a cytoplasmic tyrosine kinase protein belonging to the *src* family called *lck* (Veillete et al., 1988). Several other oncogenes have been found to be transcription factors themselves, e.g. c-*jun* and AP1 (Bohmann et al., 1987), c-*erb*A and the thyroid hormone receptor (Sap et al., 1986; Weinberger et al., 1986).

A large number of oncogenes may be acting by associating with and activating some of the proteins on the signal transduction pathway. Examples would include polyoma middle T and its association and activation of the *src* family of cytoplasmic tyrosine kinases (Courtneidge and Smith, 1983; Kornbluth et al., 1987; Cheng et al., 1988; Kypta et al., 1988) and v-*crk* and its association with unknown cellular proteins resulting in the activation of cellular tyrosine kinases (Mayer et al., 1988). Oncogenes may act by associating with known transcription factors, e.g. the association of c-*fos* and AP1 (Rauscher III et al., 1988). Oncogenes from DNA tumor viruses have been found to associate with and presumably alter the function of nuclear DNA binding proteins, e.g. the association of SV40 large T and adenovirus E1A, with the retinoblastoma gene pro-

duct, itself an "anti-oncogene" (DeCaprio et al., 1988; Whyte et al, 1988).

Overexpression of several genes involved in normal signal transduction pathways to the transcriptional apparatus have been found to confer some of the phenotypes of transformed cells. Examples include overexpression of the CSF1 gene and its receptor (Roussel et al., 1987;), of the basic fibroblast growth factor (Rogelj et al., 1988), of the insulin receptor (Wang L.-H., personal communication), and of protein kinase C (Housey et al., 1988). This too suggests that most oncogenes transform cells by using normal signal transduction pathways, and further implies that once some of the critical genes involved in the pathway are themselves induced, they may perpetuate the transformed phenotype.

In fact, considerable advances have been made in discovering various genes that are turned on by transformation, and several of them turn out to be induced in normal cells in response to normal growth signals (Sugano et al., 1987; Bedard et al., 1987; Anisowicz et al., 1987; Ryder et al., 1988; Simmons et al., 1989). It is because of these precedents that we devoted much of our effort to understanding how serum induces expression from the RSV LTR in the hope that v-*src* would utilize similar pathways to turn on the same LTR (Chapter 2).

Once the RSV LTR was shown to be responsive to a normal growth stimulus, namely serum, we could define sequence elements in the LTR that are responsible for the induction by serum, demonstrate the presence of a nuclear factor that binds to these elements, and demonstrate the serum-induction of this factor. The factor turned out to be a known transcription factor, though it was not known that it was responsive to growth signals. Further, we could show (Chapter 3) that v-*src* also stimulates expression from the RSV LTR and induces the same serum-responsive transcription factor, setting the stage for subsequent research into the mechanism of induction of this factor by v-*src*.

A genetic system for studying transcription from the RSV LTR and the effects of v-*src* on it.

The study of substrates for v-*src* has been limited to biochemical approaches due to the absence of genetic tools that can be used easily in animal cells. Encouraged by the significant advances that have been made in the study of transcription of eukaryotic genes by an effective blend of biochemical purification of transcription factors from animal cells and genetic identification of analogous activities in yeast (Wingender, 1988; Jones et al., 1988; Ptashne, 1988), we attempted to transfer the v-*src* and its effect on the RSV LTR into S. cerevisiae in an effort to establish a genetically tractable system for studying how v-*src* influences gene-expression. A similar strategy has been partially successful in delineating some of the actions of another oncogene, *ras* (DeFeo-Jones et al., 1983; Powers et al., 1984; Toda et al., 1985). My efforts in this direction are detailed in Chapter 4.

Since transcription mechanisms between yeast and animal cells appear to be very similar, the first part of this project was confined to checking if the RSV LTR directs transcription in yeast (the answer is "yes") and if similar transcriptional elements were used in yeast as in mammalian cells (again a "yes"). The second part involved the introduction of v-*src* into the yeast. It had already been shown that v-*src* can be expressed in yeast, where it retains its strong tyrosine kinase activity and its subcellular localization (Kornbluth et al., 1987; Brugge et al., 1987). However, the over-expression of v-*src* is toxic to the yeast, and we had to devise strategies for expressing minimal quantities of v-*src* and examining its effects on the RSV LTR. The final answer is not in yet, but v-*src* may be stimulating expression from the RSV LTR in yeast and it may be possible to use this genetic system for defining substrates of v-*src* important for control of gene expression.

In the epilogue, I allude to one of the clones of yeast expressing v-*src* which exhibits a phenotype that is consistent with "schmoo" formation. Schmoos are normally formed by yeast when they are exposed to mating factor of the opposite mating type. The response by yeast to mating factor is intensively studied as a model of signal transduction, and has been shown to utilize an adrenergic receptor-like receptor and alpha, beta and gamma subunits of G proteins (Nakayama et al., 1985; Hagen et al., 1986; Nakafuku et al., 1987; Dietzel and Kurjan, 1987; Whiteway et al., 1989). It may be possible that this clone of yeast has a mutation at another locus which enables v-*src* to turn on the mating factor signal transduction pathway in the absence of mating factor. Should this prove to be true, then this clone of yeast can give us another route to isolating genes on the signal transduction pathway from v-*src* to the nucleus.

CHAPTER 2

In this chapter we address the induction of the RSV LTR in untransformed 3Y1 by serum. We show that serum induces transcription from the RSV LTR, that mutations in two CCAAT boxes in the LTR abolish the serum-responsiveness and that the CCAAT boxes bind to a serum inducible nuclear factor which fails to bind when the CCAAT boxes are mutated. The CCAAT box binding factor appears to belong to the CTF/CP class of factors.

Transcription from the RSV LTR has been studied extensively. The presence of an enhancer in the 5' part of the LTR has been defined by linking it to a heterologous promoter and by mutagenesis both in the context of the natural sequences and when the enhancer drives expression from a heterologous promoter (Gorman et al., 1982b; Luciw et al., 1983; Laimins et al., 1984; Cullen et al., 1985; Weber and Schaffner, 1985; Norton and Coffin, 1987; Gowda et al., 1988). The major part of the enhancer maps to the absolute 5' end of the LTR (Fig. 2), though some enhancer activity has also been noted immediately 3' to the *Sph* I site. Mutagenesis studies have also defined essential promoter elements in the LTR. The minimal sequence required for transcription from the LTR, in vivo and in vitro, appears to be confined to the TATA box which is at position -25 relative to the transcription "start" site (Yamamoto et al., 1980; Mitsialis et al., 1983; Gilmartin and Parsons, 1983).

Nuclear proteins that bind to the LTR have also been studied extensively (Sealey and Chalkley, 1987; Karnitz et al., 1987; Goodwin, 1988; Ryden and Beemon, 1989). It appears that there are two major DNA binding proteins that bind to the RSV LTR. One of these binds over the classical enhancer, and the second binds immediately downstream from the *Sph* I site. Arrigo et al. (1987), and Carlberg et al. (1988) have also

demonstrated the existence of an internal enhancer in the *gag* region of the intact avian retrovirus which may influence expression from the 5' LTR. Further, they have shown that the CCAAT binding protein C/EBP from rat liver extracts (Landschulz et al., 1988) footprints on both the *gag* enhancer and the enhancer in the 5' end of the LTR (Carlberg et al., 1988; Ryden and Beemon, 1989). Hatamochi et al. (1988) and Maity et al. (1988) reported the presence of a CCAAT binding protein that, unlike C/EBP, is composed of two subunits. The factor, CBF, stimulates transcription from the RSV LTR in vitro and footprints over regions -146 to -121 and -82 to -56 of the LTR.

Not much is known about environmental signals that influence transcription from the RSV LTR. Temin (1967) reported that while RSV can infect chicken embryo fibroblasts (CEF) in the absence of serum, virus production is not resumed until after the addition of serum. Leong et al. (1972) showed that once the pH of the medium was adjusted by the addition of $NaHCO_3$, virus production from a RSV infected culture of CEF is not dependent on the presence of serum. Humphries and Temin (1972, 1974) demonstrated that once virus production has started in RSV infected CEF, serum deprivation had no effect on the synthesis of viral RNA and proteins. In contrast, in uninfected CEF, the production of endogenous viral RNA and protein was stimulated upon addition of serum to a serum-deprived culture (Chen et al., 1974). Endogenous retroviruses have LTRs that resemble the RSV LTR in general structure, but are shorter in the U3 region and lack the enhancer (Hughes, 1982).

All the results given above could be reconciled if it is hypothesised that in the uninfected (untransformed) cell, expression of a gene linked to the RSV LTR is dependent on serum, while transformation of a cell by v-*src* makes the LTR serum-independent. In this chapter we prove that the first half of the hypothesis is correct.

MATERIALS AND METHODS

Cells and culture conditions.

3Y1 is an immortalised, untransformed line of rat fibroblasts. They were grown in Dulbecco's modified Eagle medium (DEM) containing 5% calf serum (5% CS DEM).

Oligodeoxynucleotides.

The oligos used were as follows :

ANDU2 : 5'GATCCGCATGCCGATTGGTGGAAGTAAGGTGGTACGATCGA3'.

ANDU3 : 3'GCGTACGGCTAACCACCTTCATTCCACCATGCTAGCTCTAG5'.

ANDU5 : 3'GCGTACGGCTGGCCACCTTCATTCCACCATGCTAGCTCTAG5'.

ANDU4 : 5'CCGA$^{TT}_{CC}$GGT$^{GG}_{CC}$A$^{A}_{C}$GTAAGGT$^{GG}_{CC}$TACG3'

ANDU80 : 5'ATGGATTTTACGAACCA3'.

ANDU120: 5'AGTAAGGTTTTACGATCG3'.

ANDU140: 5'TGCCGATTTTTGGAAGTA3'.

Hsp70 : 5'TTCTTTTCTTTCTTCCCTTCTGAGCCAATCACCGAGCGCCCTAC3'

3'AAGGGAAGACTCGGTTAGTGGCTCGCGGGATG5'

cHa-ras: 5'TTCTTTTCTTTCAATGGCGCGCAGCCAATGGTAGGCCGCCCTAC3'

3'TTACCGCGCGTCGGTTACCATCCGGCGGGATG5'

H1-TF2 : 5'TTCTTTTCTTTCCTAGGTGATGCACCAATCACAGCGCGCCCTAC3'

3'GATCCACTACGTGGTTAGTGTCGCGCGGGATG5'

Ad2-NF1: 5'TTCTTTTCTTTCTTTGGATTGAAGCCAATATGATAATGCCCTAC3'

3'AAACCTAACTTCGGTTATACTATTACGGGATG5'

SV40core: 5'AGCCTGGGGACTTTCCACACCCTAACTGACA3'

3'TCGGACCCCTGAAAGGTGTGGGATTGACTGT5'

Plasmids.

RSVCAT (Gorman et al., 1982b) is a plasmid that has the U3, R, and U5 regions of the Rous sarcoma virus Long terminal repeat along with the 3' intergenic region between the v-*src* and the LTR linked upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene (Fig. 2). The resulting plasmid has been shown to direct the expression of the CAT gene using transcripts initiating at the correct initiation site in the RSV LTR. RSVNEO is a construct similar to RSVCAT that has the same RSV fragment driving the bacterial neomycin phosphotransferase gene. ΔSMCAT was made by cutting RSVCAT at unique *Sph* I and *Mst* II sites, blunting with Klenow, ligating with T4 DNA polymerase selecting against parental plasmid by cutting with *Sph* I and *Mst* II and transforming E. Coli. ΔRSCAT was made by cutting RSVCAT with *Eco* RI, blunting, ligating with *Sph* I linkers, digesting with *Sph* I and *Hind* III, isolating the 80 bp *Sph* I (formerly *Eco* RI at -51 relative to the RSV transcription start site) to *Hind* III fragment and cloning it into the large *Sph* I to *Hind* III fragment obtained from RSVCAT. 114BgCAT was made by partial digestion of RSVCAT by *Pvu* I, isolating the full length linear plasmid from an agarose gel, blunting it and ligating *Bgl* II linkers followed by selection of the correct construct by transforming E. coli to ampicillin resistance. 51BgCAT was made similarly by partial digestion of RSVCAT with *Eco* RI and ligating *Bgl* II linkers. 114BgCAT was cut at unique sites by *Bgl* II and *Mst* II ,blunted , ligated, cut once again with the same restriction enzymes and then transformed into E. Coli to produce ΔPMCAT. ΔPSCAT was made similarly by using *Sph* I instead of *Mst* II.

p18LTR was made by cloning the 440 bp *Pst* I - *Eco* RI fragment from a molecular clone of RSV 29 (Dutta et al., 1985) into the blunted *Xba* I site in the polylinker of PEMBL18 (Dente et al., 1983) and screening for inserts in both orientations. pC

contains the 90 bp *Eco* RI - *Sph* I fragment of the LTR cloned between the blunted *Xba* I and the *Sph* I sites in the polylinker of PEMBL18 and was derived from one of the orientations of p18LTR by *Sph*I digestion and ligation. pB contains the *Sph* I - *Mst* II fragment of the RSV LTR cloned between the blunted *Bam* HI and the *Sph* I sites of PEMBL18 and was also obtained from p18LTR by digestion with the appropriate enzymes. p18SV2 contains the SV40 enhancer (SV40 nucleotide 108-299) in a *Taq* I - *Kpn* I fragment from pXS16 (Fromm and Berg, 1982) cloned between the *Acc* I and *Kpn* I sites of PEMBL18.

The point mutations were made using a MUTAGENE kit (Bio-Rad). E. coli CJ236 (*dut, ung*) was transformed with pC and uracil containing single stranded DNA rescued by superinfecting the transformants with helper phage IR1gfip. pC80, 120 and 140 were made by using mutagenic oligos ANDU80, 120 and 140, respectively, on this template to make the second strand. The reaction products were used to transform E. coli MV1190 (dut^+, ung^+; destroys the uracil containing parental strand) to ampicillin resistance. Candidate colonies were picked by differential hybridization of colony lifts to the mutagenic oligos and the mutations were confirmed by sequencing miniprep DNAs using the universal primers. pC140 was used, instead of pC, as the starting plasmid and the above process repeated with ANDU80 and 120 to produce pC814 and pC1214 respectively. pC134 was the only mutant that we could obtain using the degenerate oligo ANDU4 in the above procedure and screening for mutants by directly sequencing several miniprep DNAs.

pm80CAT, pm140CAT and pm814CAT were created by isolating the 100 bp *Bam* HI - *Sph* I fragment from pC80, pC140 and pC814 respectively and ligating it into the 5 kbp *Bgl* II -*Sph* I fragment from 51BgCAT. ΔSM80CAT, ΔSM140CAT and ΔSM814CAT were derived from pm80CAT, pm140CAT and pm814CAT respectively,

by cutting the parental plasmids with *Sph* I and *Mst* II, blunting with Klenow and ligating.

pAD5 was made by cloning the ANDU2/ANDU3 oligomer into the *Bam* HI site of pUC18. pAD5pm1 was made by hybridizing ANDU2 with ANDU5 and cloning the mismatched oligo into the *Bam* HI site of pUC18, followed by sequencing miniprep plasmid DNAs from a few colonies with the universal primer to screen for the mutant.

Transfections.

For the transient transfection assays, the indicated amounts of plasmids were dissolved in 250 μl of 25 mM HEPES (pH 7.15) and 250 mM $CaCl_2$. This was vortexed continuously while 250 μl of a solution containing 280 mM NaCl, 1.5 mM Na_2HPO_4 and 25 mM HEPES (pH 7.15) was added drop-wise. The precipitate was allowed to form for 20' at room temperature and was then added to a 60 mm tissue culture dish containing a subconfluent layer of 3Y1 fibroblasts and 5 ml of fresh 5% CS DEM. Six hrs. later the medium was removed and replaced with 0% DEM (DEM with no serum) after washing the cells with Tris-buffered-saline (25mM Tris HCl pH 7.4, 137 mM NaCL, 20 mM KCl, 1.5 mM Na_2HPO_4 and 0.1% w/v glucose). Forty-eight hrs. after serum-starvation, the medium was supplemented with calf serum to 10% where indicated.

For making stable cell lines, 5 μg RSVNEO was transfected into 3Y1 cells in the same way as above. After washing the cells, they were fed with 5% DEM. 48 hrs. later, the cells were split 1:3 and put under selective pressure (400 μg/ml G418). G418 resistant colonies appeared in 2 wks. and these were pooled for subsequent assays.

CAT Assays.

These were done essentially according to Gorman et al. (1982a). The cells were washed thrice with 10 ml Tris-buffered-saline, harvested by scraping with a rubber

policeman, and lysed in 100 μl of 0.25 M Tris HCl (pH 7.8) by 3 cycles of freeze thawing. The protein content in the lysate was assayed by the Bio-rad dye binding assay. 50 - 70 μg of protein was used for each assay which was carried out in 100 μl of 0.25M Tris HCl (pH 7.8), 33 μl of 1.0 M Tris HCl (pH 7.8), 20 μl of Acetyl CoA (3.5 mg/ml) and 2.5 μl of ^{14} C Chloramphenicol (0.25 μCi; 60.0 mCi/mmol). The reactions were carried out for 40 mins. at 37^{o} C, stopped by extracting in 1 ml of ice cold Ethyl Acetate which was then dried down and dissolved in 30 μl of Ethyl Acetate. The reaction products were separated by Thin layer chromatography on silica gel using a mixture of chloroform : methanol as 95 : 5. The positions of the chloramphenicol and acetylated chloramphenicol were determined by autoradiography, the spots were excised and counted by liquid scintillation and the percentage chloramphenicol acetylated was calculated. Preliminary experiments had shown that the acetylation of chloramphenicol proceeds in a linear fashion under the conditions indicated.

S1 Nuclease Assays.

Total cellular RNA was prepared from the transfected cells by the method of Cox (1968). 20 μg of total RNA was mixed with gel purified, end labeled probe (100 000 cpm) in 20 μl of a solution containing 80 % formamide, 400 mM NaCl, 10 mM PIPES (pH 6.4) and 1 mM EDTA. The mixture was heated to 65^{o} C for 10 mins. and the probe and RNA allowed to anneal at 45^{o} C for 16 hrs.. This was then diluted to 200 μl containing 250 mM NaCl, 30 mM NaAcetate and 1 mM $ZnSO_4$ and digested with 100 units of S1 enzyme at 37^{o} C for 1 hr.. The reaction was stopped using 600 μl of cold ethanol to precipitate the nucleic acids which were then resolved on a 4% acrylamide (30% acrylamide : 0.8% bis-acrylamide) - urea (50%) gel with 1x TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA).

Slot blots, Northerns and Nuclear run-on Assays.

These were essentially performed as in Sugano et al. (1987) and Stoeckle et al. (1988). For slot blots we used 3 μg (1 μg where indicated) of total RNA and probed with nick translated pRSVNEO. Northerns were performed with 5 μg of total RNA run on a formaldehyde containing agarose gel and transferred to a nylon membrane (Zeta-bind). For the run-on assay we blotted 1 μg/slot of the *Hind* III to *Nco* I fragment of RSVNEO containing the neomycin phosphotransferase coding portion, and 5 μg/slot each of pFos (the 1.0 kb *Pst* I fragment of murine v-*fos* cloned into pUC 18, Curran et al., 1982), pTub (1.7 kb chicken beta tubulin cDNA cloned into the *Pst* I site of pBR322, Cleveland et al., 1980), p28S (4.8 kb *Sal* I - *Eco* RI fragment of mouse 28S rRNA genomic DNA clone in pBR322, Tiemeier et al. 1977), pUC18 and ϕX174 DNA. The assay itself was performed as described in Friedman et al.(1986).

Nuclear extracts.

These were prepared according to Dignam et al. (1983) as modified by Prywes et al. (1986). Briefly about 10^7 cells were washed thrice with Tris-buffered-saline on ice and harvested by scraping with a rubber policeman. The cells were swollen in Buffer A (10 mM Tris HCl pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1mM PMSF and 1 % Trasylol), broken by 25 strokes of a Dounce homogenizer with a type B pestle, the nuclei were collected and then extracted in 100 - 150 μl of Buffer C (300 mM KCl, 20 mM Tris HCl pH 8.0, 25 % v/v glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF and 1 % Trasylol) for 30 mins.. After the extracts were made, they were dialysed against 500 - 1000 volumes of BC100 (100 mM KCl, 20 % v/v glycerol, 0.2 mM EDTA, 0.5 mM DTT, 20 mM Tris HCl pH 8.0, 1 mM PMSF, 1 % Trasylol) across a Millipore VS filter (0.025 μm diameter pore size).

Gel mobility shift assays.

Usually 10 μg of nuclear extract was preincubated in a 24 μl volume of 10 mM Tris HCl pH 7.6, 1 mM DTT, 5 % v/v glycerol, 1 mM $MgCl_2$, 4 μg poly dI-dC/dI-dC, 1 μg of plasmid DNA and 60 mM of KCl (or KCl + NaCl) for 10 mins. at 25^o C. 1 ng probe (100,000-200,000 cpm) in 1 μl volume, labeled by T4 polynucleotide kinase or by end filling with Klenow, was added and incubation continued for 20 mins. The whole mixture was loaded on a 4 % acrylamide gel (30 % acrylamide : 0.8 % bis-acrylamide) with 0.25X TBE and electrophoresed at 180 V for 90 mins. at 4^o C.

Exonuclease III assay.

These were done according to Wu (1985). The DNA binding conditions were the same as described above, except that 20 μg of nuclear protein was used. At the end of the binding reaction, Exonuclease III was added to the required concentrations and incubated for 15 mins. at 30^o C. The reaction was stopped by adding an equal volume of a solution containing 40 mM Na_2EDTA and 2 % SDS followed by phenol extraction and chloroform extraction. The products were precipitated with ethanol, denatured in 25 μl of sequencing gel buffer and resolved by electrophoresis on a 16 % acrylamide (acryl : bis as 30 : 0.8) - urea (50 %) gel with 1X TBE.

RESULTS.

Preliminary observations.

We discovered the serum-responsiveness of the RSV LTR in the course of attempts to compete for transcription factors binding to the RSV LTR in vivo. Transient transfection assays were done on untransformed 3Y1 fibroblasts using a plasmid referred to as 5'+CAT. This plasmid contained a region from the 3' end of RSV spanning the *Pst* I site (-491 relative to transcription start site in the LTR) to *Eco* RI site (at -51), ligated in the correct orientation upstream from the minimal early promoter of SV40 driving the CAT gene. The presence of the fragment from the RSV (containing most of the U3 region except for the 3' end containing the TATA box) stimulated expression from the SV40 minimal promoter (containing only the A/T box and the Sp1 binding sites) by 5 fold. When the same *Pst* I to *Eco* RI fragment of the LTR inserted into pEMBL 18 (p18LTR) was used in competition in vivo against 5'+CAT, a 19:1 molar ratio of the competitor to the CAT plasmid resulted in a decrease in CAT activity to 20% of the activity from the uncompeted 5'+CAT. Our conclusion from this was that the U3 region contained in 5'+CAT acted as a transcriptional enhancer, and that it did so by binding sequence-specific DNA-binding proteins, because the enhancing activity could be competed away by the enhancer in p18LTR.

The observation that the competition was best seen when the transfections were done with a confluent layer of 3Y1, made us examine if the growth state of the cells was important for the competition. To this end we performed the competition experiments with a sparse culture of 3Y1 which were either allowed to grow normally after transfection or were made stationary by depriving them of serum. To our surprise, the deprivation of serum not only allowed more effective competition by p18LTR, but also substan-

FIGURE 1. Serum induces transcription from the RSV LTR in the absence of new protein synthesis.

(A) (Upper panel) S1 Nuclease analysis of total RNA from 3Y1 transfected with RSVNEO and RSVCAT. "-": cells in 0% DEM for 48 hrs.. "+": cells in 0% DEM for 48 hrs. followed by addition of calf serum to 10% (v/v); incubation was continued for another 6 hrs.. The probes were RSVNEO and RSVCAT, cut and labelled at an unique *Nco* I site in the NEO and CAT gene respectively. The sizes of the fragments protected by correctly initiated transcripts are 950 bases for RSVNEO and 577 bases for RSVCAT.

(B) (Lower panel) Induction of NEO RNA in 3Y1 carrying RSVNEO stably integrated in the genome. Serum was added to 10% to cultures starved of serum for 48 hrs., total RNA was harvested at the times indicated and 3 μg of RNA was used per slot in the slot blots (S). In parallel plates, cycloheximide was added to 50 μg/ml before adding serum and in this case 1 μg of RNA was used per slot (CH+S). The slot blots were quantitated by densitometry, the NEO RNA levels at the various time points were normalized to the 0 hr. (S) value and plotted.

-
+
neo
cat
1057
770
612
495

EFFECT OF SERUM
FOLD STIMULATION OF MRNA
0
5
10
0
5
10
15
20
HOURS
Serum
Cycloheximide+serum
0 1 6 12 24
S
CH+S

tially decreased the CAT activity even in the uncompeted cultures. This suggested, (a) that the presence of serum increased the level of some limiting factor that interacts with the RSV LTR upstream of the *Eco* RI site at -51 and is responsible for the "enhancer" activity of the same, and (b) that serum was required for the "enhancer" activity of the RSV LTR. We decided to examine both these issues directly.

Serum stimulates the level of transcripts initiating at the RSV LTR without requiring new protein synthesis.

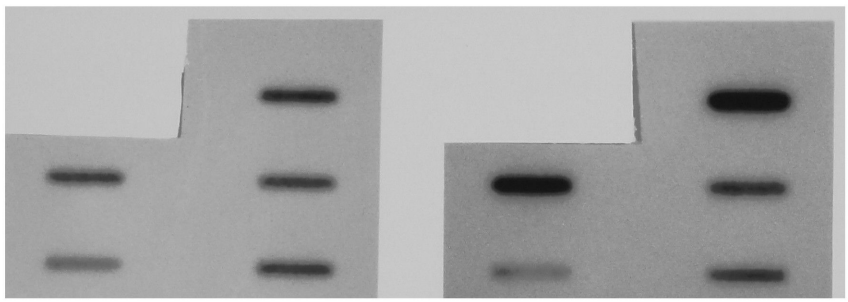
RSVCAT (Gorman et al., 1982b) and RSVNEO contain identical parts of RSV, namely the 3' intergenic region between v-*src* and the 3' LTR, the whole of U3, the R region of the LTR and part of U5 (Fig. 2), ligated upstream from the bacterial chloramphenicol acetyl transferase (CAT) gene or the neomycin phosphotransferase (NEO) gene (respectively) such that transcripts initiating in the LTR continue into the CAT or NEO gene and are processed at the 3' ends of these genes at an SV40 polyadenylation site.

To test if the transcripts initiating in the RSV LTR were induced by serum, a mixture of 5 μg RSVCAT and 5 μg RSVNEO was precipitated onto immortalized rat fibroblasts 3Y1; after 6 hrs., the plates were washed twice with Tris-buffered-saline, and the medium replaced with DEM containing no calf serum (0% DEM). After 48 hrs., calf serum was added to half the plates to 10% (v/v), and 6 hrs. later total RNA was isolated from the serum depleted cultures (Fig. 1A lane -) and from the serum stimulated cultures (lane +). As shown in Fig. 1A the steady state levels of accurately initiated transcripts as measured by S1 nuclease analysis were stimulated by serum about 5x for both RSVCAT and RSVNEO. This implies that the serum responsive signals are likely to be in the RSV LTR parts of the two plasmids.

A line of 3Y1 containing stably integrated copies of RSVNEO was created by transfecting 3Y1 with linearized RSVNEO DNA and selecting for G418 resistant

FIGURE 1.

(C) Nuclear run on transcripts were prepared from the cell line used in (B) 48 hrs. after serum deprivation (0) and 1 hr. after addition of serum to 10%. The probes used are shown in the right hand panel and described in MATERIALS & METHODS.



0

Serum
(1hr)

NEO
FOS TUB
28S PUC18

colonies. About 20 such colonies were pooled to create the cell line. When this cell line was starved of serum for 48 hrs., and then serum was added to 10% (v/v) to some of the cultures, the time course of induction of NEO transcripts (Fig. 1B) shows that maximum induction is reached in 1 hr., remained high for 5 hrs. and then declined slowly. S1 nuclease analysis (data not shown) revealed that the NEO transcripts were initiated at the correct site in the LTR.

In a parallel experiment, 3Y1 stably transformed with RSVNEO, was starved of serum, pretreated with cycloheximide at 50 μg/ml, and then treated with serum. As shown in Fig. 1B, cycloheximide treatment alone increased the steady state NEO RNA level twofold, and serum still stimulated the RNA level about fivefold above baseline despite the presence of an inhibitor of protein synthesis.

Nuclear run-on assay using this cell line (Fig. 1C) shows that the effect of serum on the steady state message from RSVNEO could be accounted for mostly by the stimulation of transcription from the RSV LTR. The presence of transcripts hybridizing to pUC18 indicates that there is some run through transcription from the NEO transcription unit and perhaps upstream cellular promoters into the adjoining pBR322 sequences. However, the NEO transcription signal is detected by hybridization to a NEO specific DNA fragment which is devoid of any DNA that could hybridize to this pBR322 background. Furthermore, no background signal is detected using ϕX174 DNA as a negative control (data not shown).

The serum responsiveness of the RSV LTR maps primarily to two CCAAT box sequences.

The structure of the RSV LTR used in RSVCAT is indicated in Fig. 2. A series of deletions were made using convenient restriction enzyme sites as shown in Fig. 3. 10 μg

FIGURE 2. The RSV LTR and point mutations made in it.

The structure of the RSV LTR at the 3' end of the provirus is shown. The *Pvu* II to *Bst* NI (converted into a *Hind* III site) is contained in RSVNEO and RSVCAT. The start site of transcription used by the virus is indicated as +1 and all other restriction sites shown relative to it. The positions of the TATA box and the classical enhancer are indicated. RI: *Eco* RI.

The lower half of the figure shows the sequence of part of the LTR which is cloned into pEMBL 18 to give pC. The lines below indicate the mutations made in this fragment (only the lower strand is represented) and the names of the corresponding plasmids.

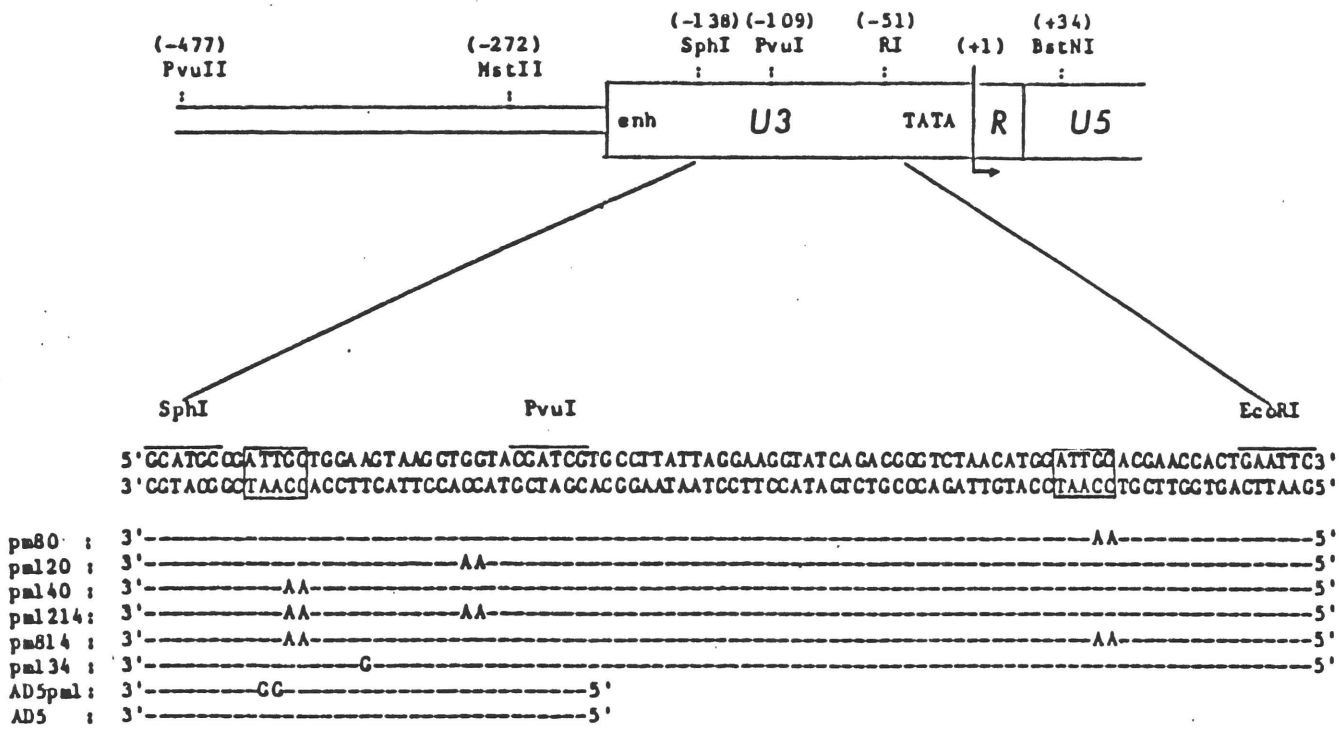

(-477)
PvuII
(-272)
MstII
(-138)
SphI
(-109)
PvuI
(-51)
RI
(+1)
(+34)
BstNI
enh
U3
TATA
R
U5
SphI
PvuI
EcoRI
5'GCATCCGCATTGCTGCAAGTAAGGTGGTACCATCGTGCCTTATTAGGAAGGCTATCAGACGCGTCTAACATGCATTGCACGAACCACTGAATTC3'
3'CGTAGGCGTAACGACGTTCATTCCACCATGCTAGCACGGAATAATCCTTCCATAGTCTGCGCAGATTGTACGTAACGTGCTTGGTGACTTAAG5'
pm80 : 3'----------AA----------5'
pml20 : 3'----------AA----------5'
pml40 : 3'----------AA----------5'
pml214: 3'----------AA----------AA----------5'
pm814 : 3'----------AA----------AA----------5'
pml34 : 3'----------G----------5'
AD5pml: 3'----------GG----------5'
AD5 : 3'----------5'

FIGURE 2

of RSVCAT and each of the deletion constructs shown were transfected into parallel cultures of 3Y1. After 6 hours of exposure to the DNA precipitate, each culture was washed twice with Tris-buffered-saline and placed in 0% DEM for 48 hrs.. Calf serum was added to 10% (v/v) and 12 hrs. later the cells harvested, lysed, and equal quantities of protein were used to measure the amount of CAT activity derived from RSVCAT and each of the deletion derivatives. For each experiment, each plasmid was transfected into at least 2 different plates of cells and the CAT activity determined separately. The CAT activity obtained from RSVCAT was considered to represent the 100% level of activity obtained from the intact LTR upon serum stimulation. The CAT activities obtained from each of the other plasmids were expressed as a percentage of the activity obtained from RSVCAT. The results are presented as a mean +/- standard deviation with the number of independent transfections shown in parentheses.

It can be seen from the results in Fig. 3 that ΔSM deletion which deletes the conventional RSV enhancer did not significantly diminish the serum induced CAT activity relative to RSVCAT. However, extending the deletion to the *Pvu* I site (ΔPMCAT) caused the CAT activity to drop to 40% the level relative to RSVCAT. Extending the deletion still further to the *Eco* RI site (ΔRMCAT) caused the activity to drop to 2% relative to RSVCAT. These results indicate that the serum responsiveness of the RSV LTR resides mostly in two segments : *Eco* RI - *Pvu* I and *Pvu* I - *Sph* I. This is confirmed by the result with ΔRSCAT where removal of both the serum responsive elements decreases the CAT activity to 6.5% the level of RSVCAT. It is of interest to note that the deletion in ΔPSCAT is not as effective in decreasing CAT activity as the deletion in ΔPMCAT, suggesting that the enhancer in the *Sph* I - *Mst* II fragment (SM fragment) can partially compensate for the absence of the *Pvu* I - *Sph* I element (PS element).

FIGURE 3. Serum responsiveness of the RSV LTR maps primarily to two CCAAT sequences.

CAT activities obtained by transient transfection of RSVCAT and its derivatives into 3Y1 (as described in the text) is shown along with the names of the plasmids and schematic representations of the mutations in the plasmids. RSV for RSVCAT, SM for ΔSMCAT, pm80 for pm80CAT, SM80 for ΔSM80CAT etc.. Restriction enzyme sites indicated for RSVCAT are R: *Eco* RI, P: *Pvu* I, S: *Sph* I, M: *Mst* II (Fig. 2). In the schematics, gaps indicate deletions made using the restriction enzyme sites shown, and dots indicate point mutations (Fig. 2). The CAT activities are normalized to RSVCAT (100%); the number following the + is the standard error of the number of independent observations shown in parenthesis.

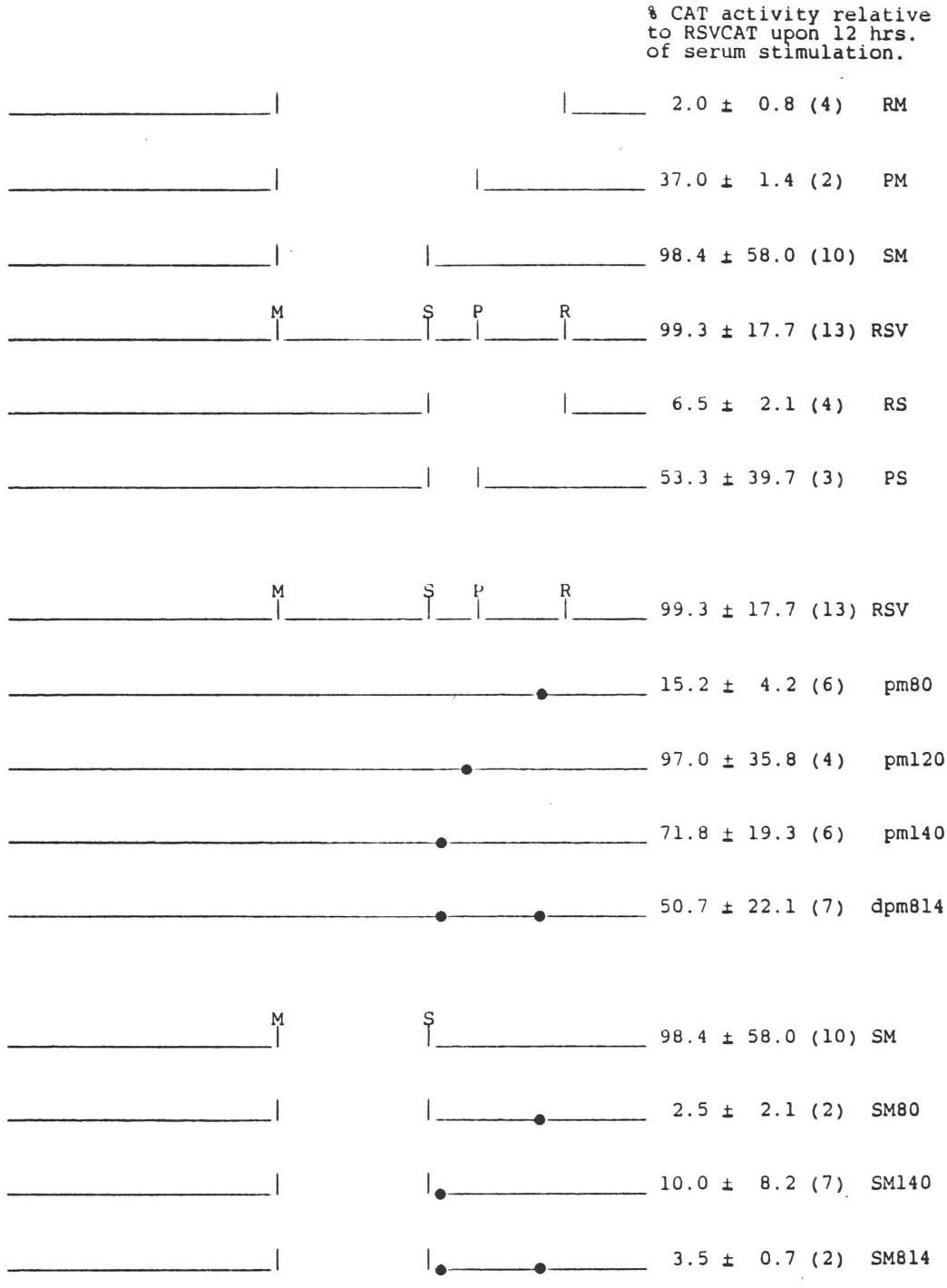

% CAT activity relative to RSVCAT upon 12 hrs. of serum stimulation.
2.0 ± 0.8 (4) RM
37.0 ± 1.4 (2) PM
98.4 ± 58.0 (10) SM
M S P R
99.3 ± 17.7 (13) RSV
6.5 ± 2.1 (4) RS
53.3 ± 39.7 (3) PS
M S P R
99.3 ± 17.7 (13) RSV
15.2 ± 4.2 (6) pm80
97.0 ± 35.8 (4) pm120
71.8 ± 19.3 (6) pm140
50.7 ± 22.1 (7) dpm814
M S
98.4 ± 58.0 (10) SM
2.5 ± 2.1 (2) SM80
10.0 ± 8.2 (7) SM140
3.5 ± 0.7 (2) SM814

FIGURE 3

In an attempt to define the transcriptional elements most necessary for wild type activity upon the addition of serum, we examined the *Eco* RI - *Sph* I (RS fragment) sequence (Fig. 2). The CAT assays presented above indicated that the RS fragment contained the elements that were most important for serum stimulated transcription. RNA data to be presented at the end of this section will confirm that these elements are important for both the basal level of activity in the absence of serum, and the induction of activity seen upon the addition of serum. Two CCAAT boxes were noted in the antisense strand of the RS region as shown in Fig. 2. Using oligonucleotide directed mutagenesis, we made the mutations shown in Fig. 2 and introduced the pm80, pm120, pm140 and dpm814 mutations into the RSV LTR of RSVCAT. These mutants were analyzed in the same way as the deletion plasmids and the results are presented in Fig. 3. pm80CAT produced 15% of the CAT expressed by RSVCAT, indicating that the promoter proximal CCAAT box ("80") is very important for serum responsiveness. pm120CAT showed no decrease in CAT activity while pm140CAT showed a minor decrease. However, in light of the difference between ΔPMCAT and ΔPSCAT discussed above, we made additional constructs where the mutations of pm80CAT and pm140CAT were introduced into an LTR that already has a deletion of the SM fragment. The results with ΔSM80CAT and ΔSM140CAT map the two transcriptional elements in the RS fragment to the two CCAAT boxes.

The enhancer in the SM fragment appears to have some role in the serum stimulated transcription in view of the differences between ΔPSCAT and ΔPMCAT and also pm140CAT and ΔSM140CAT. Further, the introduction of the 140 mutation into an LTR that already has a mutation in the proximal CCAAT box (dpm814CAT) causes an increase in serum stimulated CAT activity relative to pm80CAT. This is not seen when the SM fragment is deleted (compare ΔSM814CAT with ΔSM80CAT), implying that

TABLE 1

	(−) basal level	(+) induced level	1. basal level of mutant relative to wild type	2. induction ratio of mutant relative to wild type
RSVCAT	2.5	6.3	1	1.3
RSVNEO	12.0	23.2		
△PSCAT	1.0	5.1	1.3	1.1
RSVNEO	3.6	17.2		
△SMCAT	7.5	57.2	3. n.d.	0.71
RSVNEO	8.6	92.2		
△RSCAT	1.1	2.2	0.52	0.41
RSVNEO	10.0	49.1		

The amount of CAT RNA produced from each XCAT plasmid in the absence of serum (basal level) and 2 hrs after addition of serum (induced level) was measured by transient transfection, followed by S1 nuclease analysis of the RNA. RSVNEO was used as an internal control for transfection and for serum induction. For △SMCAT, the basal level could not be detected by transient transfection. Therefore, we created a stable cell line containing △SMCAT and RSVNEO by co-transfecting the two plasmids, selecting for and pooling several G418 resistant colonies. The levels of CAT and NEO transcripts before and after addition of serum were measured with this cell line by Northern blot analysis with CAT and NEO specific probes. In all cases, the relevant signals were quantitated by densitometry and the results presented in columns 2 and 3.

1. $XCAT^{-} \div RSVNEO^{-}$ in XCAT transfection / $RSVCAT^{-} \div RSVNEO^{-}$ in RSVCAT transfection

2. $XCAT^{+} \div XCAT^{-}$ / $RSVNEO^{+} \div RSVNEO^{-}$ in same transfection as XCAT.

3. not determinable, because the induction ratio was measured using a cell line carrying △SMCAT and RSVNEO stably integrated into the genome. Hence unlike the other XCATs, the basal level of the △SMCAT plasmid could not be compared directly to the basal level of RSVCAT as determined by transient transfection assays.

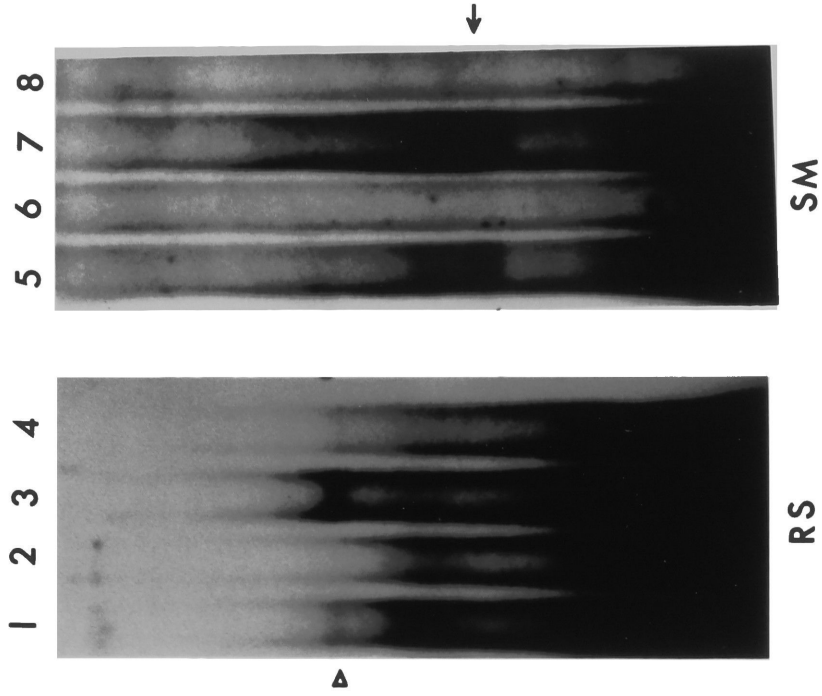
the presence of the enhancer has a stimulatory effect on the serum responsiveness of the intact LTR and this is interfered with by the distal CCAAT sequence ("140" CCAAT) in the pm80CAT construct.

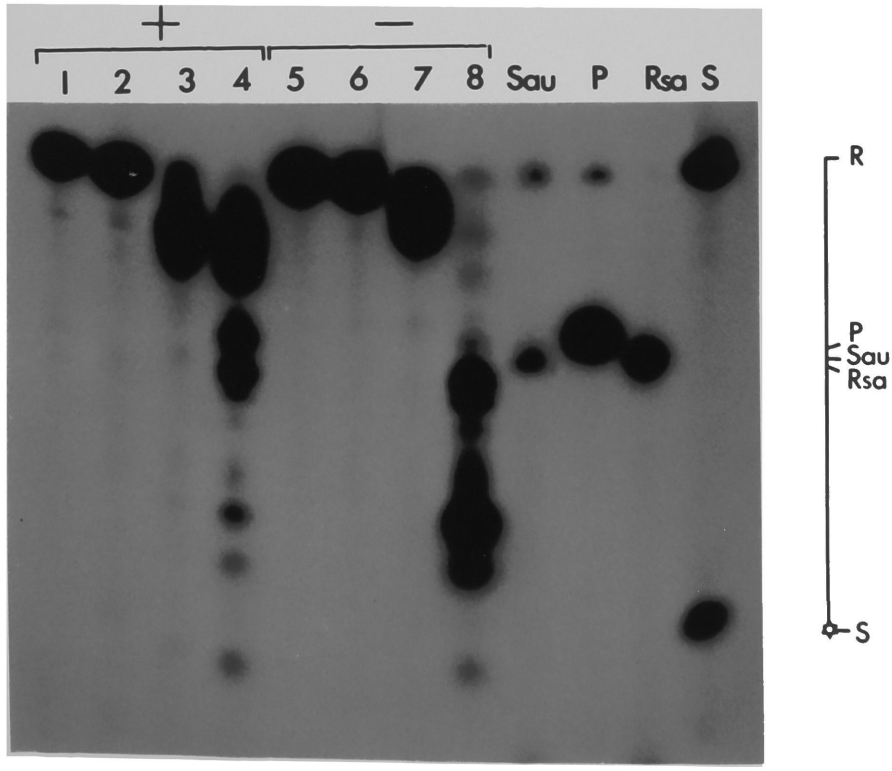
To ensure that the effects seen with the mutations were a reflection of change in the induction ratio and not merely due to changes in the basal level of expression, the experiment shown in Fig. 1A was repeated with some of the mutant CAT plasmids. 10 μg/plate of RSVCAT (or some of the mutant CAT plasmids) and 5 μg/plate of RSVNEO (as an internal control for transfection) were co-transfected into 3Y1, the cells were serum starved for 48 hrs., the plates were treated with serum (10% v/v) for 2 hrs. before total cellular RNA was purified from the cells and subjected to S1 analysis for accurately initiated CAT and NEO transcripts. The bands corresponding to the CAT and NEO transcripts were quantitated by densitometry, and the results presented in Table 1. As can be seen, a decrease in induction ratio relative to wild type was seen with ΔRSCAT and to a lesser degree with ΔSMCAT. This supports our conclusion that the serum responsiveness resides mostly in the RS fragment. The SM fragment may play a secondary role. It should be noted that the deletion of the RS fragment is also accompanied by a decrease in the basal level of activity (the activity in the absence of serum). However, if this decrease in basal level of activity was due to elements that were not responsible for the serum induction, one would not expect to see an accompanying decrease in the induction ratio: the induced level of the mutant would be decreased relative to wild type induced level by the same factor as the mutant basal level would be decreased relative to wild type basal level. The fact that this is not the case suggests that the elements in the RS fragment are responsible for both the basal level of activity and for the induction by serum. The argument is bolstered by the observation that deletion of the SM fragment has a much greater effect on the basal

FIGURE 4. Induction by serum of a nuclear factor binding to the RS fragment.

(A) (Upper picture) Gel mobility shift analysis. Left panel: probe is the *Eco* RI - *Sph* I fragment (RS) of the RSV LTR. Right panel: probe is the *Sph* I - *Mst* II fragment (SM) of the LTR. The plasmids in the reactions were:- lanes 1, 3, 5, 7: pEMBL 18; lanes 2, 4: pC (pEMBL 18 with the RS fragment cloned in it); lanes 6, 8: pB (pEMBL 18 with the SM fragment cloned in it). The nuclear extracts used were from serum starved 3Y1 (1, 2, 5, 6) or from 3Y1 treated with 10% serum for 2 hrs. after serum starvation (3, 4, 7, 8). Specific DNA-protein complexes are marked for RS (arrowhead) and SM (arrow).

(B) (Lower picture) Exonuclease III footprinting. The *Eco* RI - *Hind* III fragment of pC containing the RS fragment and the *Hind* III site from the polylinker of pEMBL 18 was labelled at the *Hind* III site by T4 polynucleotide kinase. Lanes 1-4: nuclear extract from serum-stimulated 3Y1. Lanes 5-8: no extract. Exonuclease III concentrations were 0 u/ml (1, 5), 135 u/ml (2, 6), 450 u/ml (3, 7), and 1500 u/ml (4, 8). The labelled, upper strand of the RS fragment is represented next to the gel with the positions of some restriction enzyme sites. R: *Eco* RI, P: *Pvu* I, Sau: *Sau* IIIA, Rsa: *Rsa* I, S: *Sph* I. The lanes marked with letters representing restriction enzymes are size markers created by cutting the labelled DNA with the corresponding enzymes.


5 6 7 8
SM
1 2 3 4
RS


+
−
1 2 3 4 5 6 7 8 Sau P Rsa S
R
P
Sau
Rsa
S

level (it could not be detected by transient transfection assays) without an equivalent effect on the induction ratio. Thus a decrease in the basal level would not automatically cause an equivalent decrease in the induction ratio, unless the element responsible for the basal level of activity was also responsible for the serum-responsiveness. We are currently extending this analysis to the plasmids carrying the point-mutations.

Till the data from the point mutations is available, it is not possible to definitively say that the same elements in the RS fragment that are responsible for the basal level of transcription are also responsible for the induction. Theoretically, it could be envisaged that the RS region contains elements responsible for basal activity, like the CCAAT elements, and other elements that are responsible for the induction. However, the data presented below shows that the only nuclear factors that bind to the RS region do so through the CCAAT elements, making it highly likely that the CCAAT elements are the only transcriptional elements in the RS region, and they are responsible for both the basal level of activity and the induction by serum.

Serum induces the appearance of a nuclear protein that binds to the RS fragment.

Nuclear extracts were prepared from 3Y1 starved of serum for 48 hrs., and from parallel cultures that, after serum-deprivation, were exposed to 10% calf serum (v/v) for 2 hrs. before making the extracts. Gel retardation DNA binding assay (Fig. 4A) revealed that a nuclear protein bound specifically to the RS fragment (the band marked with an arrowhead disappears when cold RS fragment is put in competition in lanes 2 and 4). Further, the abundance of the protein is increased upon exposure of the cells to serum (lane 3 compared to lane 1). In contrast, the nuclear protein(s) binding to the enhancer SM fragment (lanes 5-8) do not appear to be induced to the same extent by serum (lanes 5 and 7).

FIGURE 4.

(C) Gel mobility shift assay using labelled RS fragment (left panel) and PS fragment (right panel). PS was made by annealing oligos ANDU 2 and 3 and purifying the double-stranded oligo on a gel. The competing plasmids and oligos present in a reaction are indicated above the lanes. p18: pEMBL 18. pC: pEMBL 18 with one copy of the RS fragment cloned in it. Arrowhead: specific DNA protein complex.

p18
pC
p18+PS
p18
pC
RS (C)
PS (2/3)

The experiments using deletion constructs above showed that there were two serum responsive elements in the RS fragment separated by the *Pvu* I site. When an exonuclease III mapping was done to gain an idea of where the nuclear proteins may be binding to the RS fragment (Fig. 4B), one could distinguish two major exo III pause sites on the upper strand of the RS fragment. As can be seen on comparing lanes 4 and 8 of Fig. 4B, one pause site appears to be between the *Eco* RI and *Pvu* I sites and a second pause site appears to map just to the left of the *Pvu* I site.

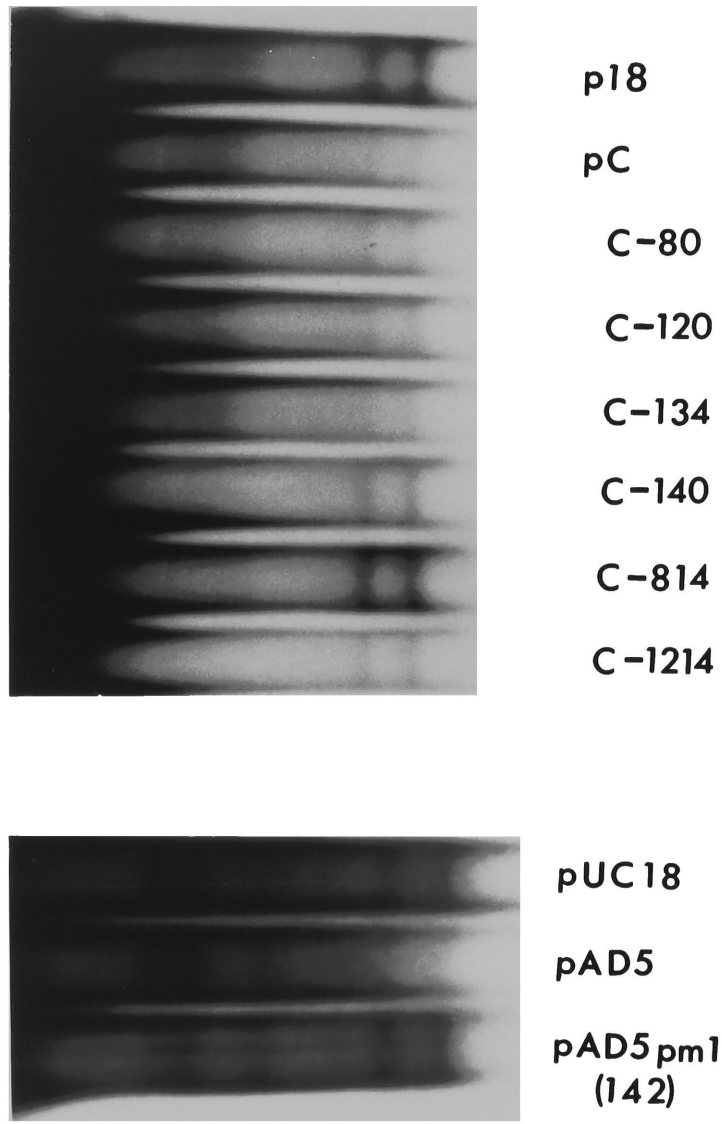
Two oligonucleotides (ANDU 2 and 3) were synthesised such that they could be annealed to form a double stranded DNA fragment that was homologous to the PS fragment. This synthetic PS fragment could compete for the factor binding to the RS fragment in a gel retardation assay (Fig. 4C), suggesting that the two serum-responsive elements in the RS fragment (only one of which is on the PS fragment) may be binding the same nuclear factor. The PS fragment could itself be shifted up in a gel retardation assay and this could be competed for by the entire RS fragment (Fig. 4C right panel) or by cold PS fragment (data not shown).

The RS fragment binding factor binds to the two CCAAT box sequences.

The point mutants made in the RS fragment that are shown in Fig.2 were each cloned into the polylinker of pEMBL18 and these plasmids were used to compete for the RS binding factor in a gel retardation assay (Fig. 5). The appearance of two bands upon gel retardation with the RS fragment (Fig. 5) or PS fragment (data not shown), as opposed to the single band seen in Fig. 4, seems to depend on the concentration of the nuclear protein extract. As can be seen, mutants that retain even one of their CCAAT boxes (pC-80, pC-140) still compete for the RS binding factor, while mutants that have lost both their CCAAT boxes (pC-814) fails to compete for the RS binding factor. This definitely demonstrates that the serum inducible nuclear protein that binds to the RS

FIGURE 5. The serum induced nuclear protein binds to the CCAAT sequences in the LTR.

Gel mobility shift assay with the RS fragment and nuclear extract from serum-stimulated 3Y1. The plasmid present in competition in each reaction is indicated above each lane, e.g. p18: pEMBL 18, C-80: pC with the pm80 mutation (Fig. 2) etc..


RS
p18
pC
C-80
C-120
C-134
C-140
C-814
C-1214
pUC18
pAD5
pAD5pm1
(142)

fragment does so through either of the two CCAAT box sequences that have already been identified as the major mediators of the serum responsiveness of the RSV LTR.

The right hand panel of Fig. 5 confirms that the PS fragment alone can compete for the RS binding factor (pAD5 has one copy of the PS oligo cloned into pUC18) and this is abolished by a mutation in the CCAAT box of the PS fragment (pAD5pm1; Fig. 2).

As pointed out in the discussion of Table 1, the RS fragment appears to contain elements that are responsible for both the basal level of transcription (in the absence of serum) and for the actual induction seen upon the addition of serum. It is theoretically possible that the two activities reside in two different elements. However, in view of the experiments done with nuclear factors, it is clear that the only factors that can be seen binding the RS fragment are CCAAT binding factors, which makes it very likely that the CCAAT elements are the only transcriptional elements in the RS fragment and they are responsible for both the basal level of activity and the induction by serum.

A CCAAT box binding nuclear factor is induced by serum in the presence of an inhibitor of protein synthesis.

The time course of the induction of nuclear CCAAT box binding factor upon serum stimulation of serum starved 3Y1 was studied. A gel retardation assay was done with labeled PS fragment using 3 μg of nuclear protein for each reaction. Two specific bands were seen which were lost on competition with pAD5 but not pAD5pm1. Densitometric scanning of the bands was performed to quantitate the amount of nuclear CCAAT binding activity seen per μg of protein at the various time points, and the results presented in Table 2, Experiment 3. The lower band (Band 1) was clearly induced upon addition of serum, with a time course that is reminiscent of the induction of RSVNEO mRNA (Fig. 1B). The upper band appeared to remain constant. Surprisingly, the amount of

Table 2. Nuclear CCAAT factor is induced by serum in the presence of cycloheximide.

Hrs. in 0%	cyclohex (μg/ml)	medium (hrs)	Nucl.extr. (μg/plate)	CCAAT binding activity specific (units/μg protein)		total (units/plate)	
				Band1	Band2	Band1	Band2
EXPERIMENT 1							
72	10	0%(2)	1.3	13.0	-	16.9	-
72	10	10%(2)	15.6	10.7	-	166.9	-
EXPERIMENT 2							
48	0	0%(2)	18.9	1.7	3.1	32.1	58.6
48	50	0%(2)	3.3	0.9	11.0	3.0	36.3
48	50	10%(2)	9.6	0.7	5.9	6.7	56.6
EXPERIMENT 3							
72	0	0%(0)	3.9	0	1.8	0	7.0
72	0	10%(1)	5.2	1.1	3.1	5.7	16.1
72	0	10%(3)	16.8	4.6	3.2	77.3	53.8
72	0	10%(6)	41.6	3.0	2.8	124.8	116.5

Cycloheximide was added to the serum-starved cultures in experiments 1 and 2 for 15 mins., at the concentrations indicated, before the addition of serum. The cells were either left in 0% DEM or calf serum was added to 10% (v/v) and incubation continued for the no. of hrs. shown in parentheses. Nuclear extracts were prepared in equal vols. of buffer per plate, and the protein concentrations determined by the dye-binding assay. Gel mobility shift analysis was done using equal quantities of nuclear protein per lane and radiolabelled PS probe. Band 1: the faster moving specific DNA-protein complex. Band 2: the slower moving DNA-protein complex. Since the probe was in excess, the amount of the probe in a complex is proportional to the amount of CCAAT- binding protein in the extract. The bands were scanned using a laser scanner and the areas under the curves measured by cutting them out and weighing them. The weights in mgs, divided by the amount of nuclear proteins used in the reaction, gives an estimate of the specific CCAAT binding activity/μg of nuclear extract protein. The validity of this measurement was tested by using different dilutions of the same nuclear extract (data not shown). Because of differences in the specific activities of the probe and other variations between the different experiments, the actual numbers in columns 5-8 can be compared in the same experiment, but not between experiments. "-": band not seen. Total CCAAT-binding-activity present per plate was obtained by multiplying the numbers in columns 5 or 6 with those in column 4.

nuclear protein obtained per plate increases 10 fold upon addition of serum within a time period that was not sufficient to allow cell division. If this is taken into account, then the comparisons in the gel shift assays, which used equal quantities of protein per lane, underestimate the actual induction of the nuclear factors per cell. Thus if one were to calculate the amounts of proteins that produce Band 1 or Band 2 on a per cell basis (last two columns of Table 2), then the induction by serum is almost 100 fold and 15 fold, respectively.

In this context, the effect of pretreating the serum starved 3Y1 with cycloheximide (Table 2, Experiments 1 and 2), before adding serum is interesting. In experiment 1, serum caused a 12 fold increase in the amount of protein in the nuclear extract. However the amount of CCAAT binding factor that gave rise to band 1 per μg of nuclear protein was approximately the same in the cells treated with cycloheximide alone as opposed to the cells treated with cycloheximide and serum. This implies that on a per cell basis (or as presented in Table 2, a per plate basis), serum stimulated the amount of nuclear CCAAT factor despite the presence of cycloheximide at concentrations that inhibit nearly 97% protein synthesis in fibroblasts (data not shown). In experiment 2, the concentration of cycloheximide is enough to inhibit 99% protein synthesis and serum still causes an increase in nuclear CCAAT binding factor per plate. Closer examination of the data reveals that while serum can induce nuclear CCAAT factor in the virtual absence of protein synthesis, the magnitude of the induction is greater when protein synthesis is permitted.

The CCAAT box binding factor that binds to the RSV LTR belongs to the CTF/CP1,2 class of factors.

Many different CCAAT box binding factors have been characterized (Dorn et al., 1987; Chodosh et al, 1988a; Landschulz et al, 1988; Santoro et al., 1988; Gil et al., 1988;

FIGURE 6. The serum induced factor binds to other CCAAT sequences.

(A) (Upper picture) Other CCAAT oligos compete for the CCAAT factor that binds to the RSV LTR. The PS fragment was labelled and a gel mobility shift assay performed using nuclear extracts from serum-stimulated 3Y1 cells. pAD5 was present in the reaction for the first lane and pAD5pm1 in all the other lanes. The oligos (and their amounts in ng) used in competition are shown above the corresponding lanes For a full description of the oligos see MATERIALS & METHODS and Fig. 7.

(B) (Lower picture) The CCAAT factor forms the same sized complex with the PS oligo and the oligos that can compete for the RSV LTR CCAAT factor. The oligos shown below the lanes were labelled and used in gel mobility shift assays with the same nuclear extracts as in (A). The plasmid used in competition in each reaction is shown above each lane.

AD5
AD5pm1
50
HSP70
200
50
H1-TF2
200
50
cHA-RAS
200
50
AD NF1
200
50
SV40
200
PS

p18SV2
pC
p18
PS
HSP70
H1-TF2
SV40

Paonessa et al., 1988; Hatamochi et al., 1988;). Some of the oligos that can bind to them are presented in Fig. 7. Double stranded synthetic oligomers were used to compete for the CCAAT factor binding to the PS fragment of the LTR. As shown (Fig. 6A), the oligos that bind the CTF/CP class of oligos, namely, the human Hsp 70 CCAAT oligo, the human histone 1 CCAAT oligo, and the cHa-ras CCAAT oligo compete for the LTR CCAAT factor effectively. On the other hand, the nuclear factor 1 (NF1) binding site from the adenovirus origin of replication competed poorly and the SV40 enhancer core oligo which binds C/EBP did not compete at all.

Since some CCAAT box factors appear to be composed of a complex of proteins (Chodosh et al., 1988a; Hatamochi et al., 1988), and since, some transcription factors form complexes with other proteins (Rauscher III et al., 1988; Shaw et al., 1989), the gel shift bands 1 and 2 could have been produced by the interaction of the LTR PS fragment with a complex of proteins only one of which is a CCAAT box binding factor. The data in Fig. 6B indicates that if a complex of proteins binds to the PS CCAAT box, at least the same sized complex also binds to the CCAAT boxes that compete for the LTR CCAAT factor (CTF/CP). It has been shown that the positions of the bands produced by the protein-DNA complex in a gel retardation assay is proportional to the sum of the sizes of the free oligo and the protein(s) binding to it (Bading, 1988). Since all the oligos used in this experiment were approximately the same size, the co-migration of the respective protein-DNA complexes implies that protein(s) of the same size are binding to each of the oligos. As a control, it can be seen that the protein that binds to the SV40 core oligo (C/EBP) produces a complex that is of a different size.

FIGURE 7. The CCAAT factor binding oligos.

Only the strand containing the CCAAT sequence (boxed in) is shown. The bases outside the CCAAT box that were the same between the oligos that bind the RSV LTR CCAAT factor and the consensus sequences developed for known CCAAT factor binding sites are shaded. "-": same as line below. Lower case letters: bases that were different 3' to the distal ("140") CCAAT box among the various DNA fragments used in the gel retardation assays. RSV140: distal CCAAT box in the RSV LTR, RSV80: promoter proximal CCAAT box in the RSV LTR, Hsp70: CCAAT box from the human heat-shock-protein 70 promoter, cHa-ras: CCAAT box from the promoter of human cellular Harvey *ras* oncogene, H1-TF2: CCAAT box from human histone-1 promoter, Ad2-NF1: Nuclear factor 1 binding site from adenovirus 2, SV40core: enhancer core sequence of SV40 72 bp repeats.

The lower half of the figure shows consensus binding sites of three types of CCAAT binding factors from human HeLa cells (Chodosh et al., 1988a) along with the methylation interference contact points. Triangles pointing up: contact point on the strand shown, pointing down: contact point on the opposite strand. Solid triangles: complete interference, open triangles: partial interference. The lowest line shows methylation interference footprint of a HeLa cell factor that binds to the RS and PS fragment of the RSV LTR.

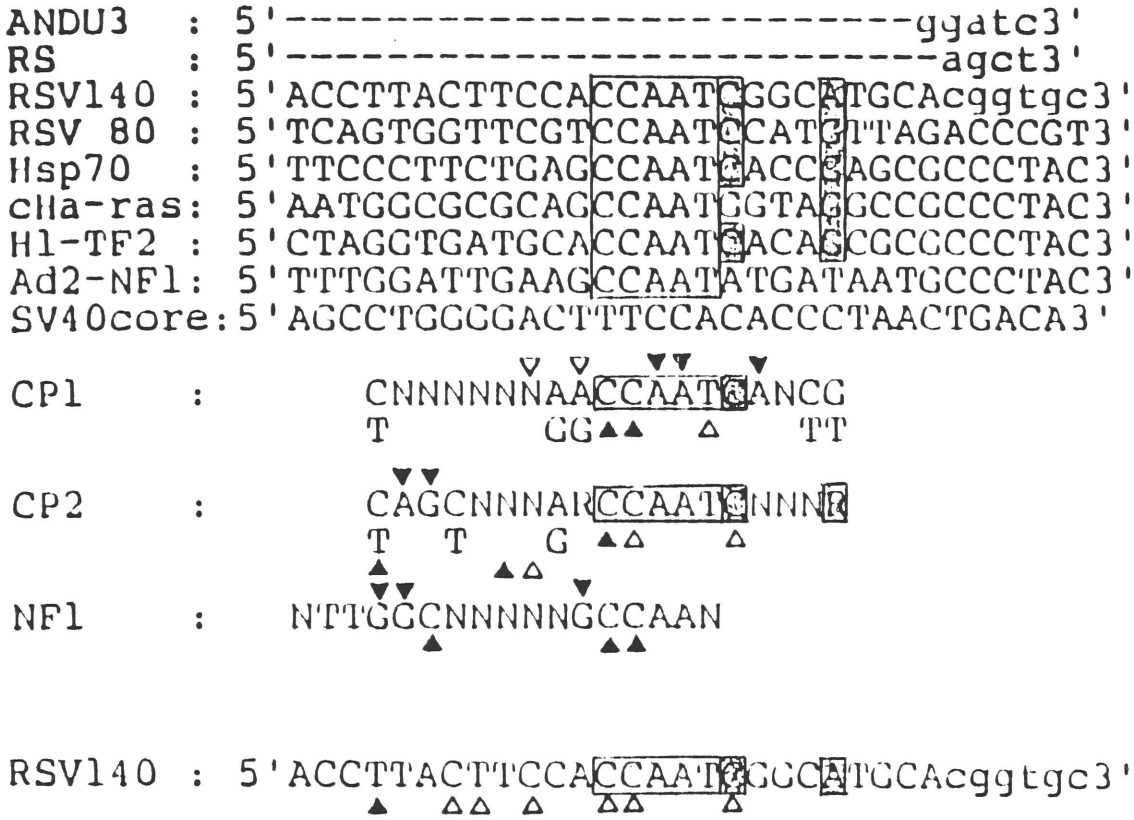

ANDU3 : 5'----------ggatc3'
RS : 5'----------agct3'
RSV140 : 5'ACCTTACTTCCACCAATCGGCATGCAcggtgc3'
RSV 80 : 5'TCAGTGGTTCGTCCAATCCATGTTAGACCCGT3'
Hsp70 : 5'TTCCCTTCTGAGCCAATGACCGAGCGCCCTAC3'
cHa-ras: 5'AATGGCGCGCAGCCAATGGTAGGCCGCCCTAC3'
H1-TF2 : 5'CTAGGTGATGCACCAATGACAGCGCCCCTAC3'
Ad2-NF1: 5'TTTGGATTGAAGCCAATATGATAATGCCCTAC3'
SV40core:5'AGCCTGGGGACTTTCCACACCCTAACTGACA3'
CP1 : CNNNNNNAACCAATGANCG
T GG TT
CP2 : CAGCNNNAICCAATGNNNN
T T G
NF1 : NTTGGCNNNNNGCCAAN
RSV140 : 5'ACCTTACTTCCACCAATGGGCATGCAcggtgc3'

FIGURE 7

DISCUSSION.

The stimulation of transcription from the RSV LTR by serum is part of a more generalized phenomenon.

Stimulation of transcription from the RSV LTR by serum adds to a list of promoters that are switched on by serum, e.g. c-*fos*, c-*myc*, c-*jun* B, actin (Ryder et al., 1988; and references therein). This may be a much more generalized phenomenon than previously suspected. Adding support to this view, is our observation that the transcription factor that mediates the serum responsiveness of the RSV LTR is not an unique factor special for the RSV LTR, but a CCAAT box binding factor that can act on many cellular promoters. The fact that serum causes a 10 fold increase in total salt-extractable nuclear proteins per cell (Table 2), adds credence to the view that the phenomenon we are observing with the CCAAT box binding factor is typical of many nuclear transcription factors. This observation is also in line with the reported burst of ^{3}H-uridine incorporation upon addition of serum to a confluent culture 3T3 fibroblasts (Todaro et al., 1965).

How does serum turn on transcription from the RSV LTR?

As in the instances cited above, the effect of serum on the RSV LTR appears to be primary, i.e. it can occur without new protein synthesis, suggesting that the transduction of the signal from serum outside the cell to the nucleus is direct and involves modification of pre-existing proteins. What could be the mechanisms involved ?

Since the induction of nuclear CCAAT box binding factor by serum can occur in the presence of cycloheximide, and mutations in the CCAAT factor binding sites in the LTR abolish the binding and decrease both the basal level of transcription in the

absence of serum, and the induction ratio upon exposure to serum, we propose that the CCAAT box binding factor mediates the primary signal from serum. One possibility is that serum induces post-translational modifications directly on the CCAAT box binding factor and activates its DNA binding activity. This would be analogous to the induction of the DNA binding activity of the heat shock factor by phosphorylation in response to heat shock (Sorger et al., 1987; Zimarino and Wu, 1987; Sorger and Pelham, 1988). It is of interest that at least one type of post-translational modification has been reported for the CCAAT box binding factor, i.e. O-glycosylation (Jackson and Tjian, 1988). In view of the serine phosphorylation of another serum responsive factor, the c-fos SRF (Prywes et al., 1988), it would be worthwhile to examine whether the CCAAT box binding factor is also phosphorylated. Any post-translational modification that is found on the CCAAT factor should be (a) modulated by serum, and (b) influence the amount of CCAAT binding activity that can be found in the nucleus for it to be implicated in the signal transduction process.

Another possibility is that serum causes post-translational modifications in another protein which interacts with the CCAAT factor. It has been proposed that at least two proteins are required to form a complex for binding to a CCAAT box (Chodosh et al., 1988a; Hatamochi et al., 1988). This has been found not to be true with cloned CCAAT box binding factors in vitro (Santoro et al., 1988), but it could still occur in vivo. It is therefore possible that serum causes a modification in an accessory protein which is essential to allow the complex to form and bind to CCAAT sequence. Serum could also modify a negative factor that is complexed with the CCAAT binding factor and thus unmask the DNA binding activity of the CCAAT factor. This would be analogous to the activation of NFkB by TPA which appears to inactivate an inhibitor of NFkB, IkB (Bauerle and Baltimore, 1988a, 1988b). These questions can be addressed by using anti-

bodies against CCAAT binding factors to study the proteins that are complexed with them in serum starved and serum-stimulated cells.

A third possibility is that serum activates a regulated pathway for the uptake of transcription factors by the nucleus. This implies the existence of active CCAAT box binding factor in the cytoplasm of the serum-starved cell. The addition of serum either causes the appearance of a signal on the factor (post-translational modification? induction of a co-factor?) that is recognized by the nuclear uptake machinery, or activates some component of the uptake machinery, or represses an inhibitor of the uptake process.

Finally, the eventual mechanism may turn out to be a combination of all or some of the above possibilities.

The above discussion dealt only with the ways in which serum can induce nuclear CCAAT box binding factor without new protein synthesis. However, as the results in Table 2 make evident, the magnitude of the induction is greater in the presence of protein synthesis, raising the possibility that serum might also stimulate the synthesis of new CCAAT box binding factor, perhaps by increasing the amount of CTF mRNA analogous to the stimulation of transcription of the AP1 gene by EGF (Quantin and Breathnach, 1988).

Is the induction by serum related to the cell cycle?

There was always the possibility that the RSV LTR is active only in one phase of the cell-cycle, and the addition of serum stimulates the expression from the LTR only because it stimulates the cells to move through the cell-cycle. We ruled out this possibility by adding aphidicolin to 5 μg/ml to the serum-starved cells before adding serum. There was no decrease in the stimulation of NEO transcripts (from RSVNEO) in the

presence of aphidicolin (data not shown), thus indicating that the RSV LTR is stimulated by the G0-G1 transition and does not require the cells to enter the S phase.

Which component of serum is responsible for the induction?

It had been reported that the serum requirement of chicken fibroblasts could be partially met by feeding the cells with high doses of insulin (Temin, 1967b). In our hands, CAT expression from RSVCAT in serum starved 3Y1 could be partially restored by insulin or insulin like growth factor 1 (IGF1) or epidermal growth factor (EGF) but not to the full extent obtained with 10% calf serum (data not shown). All of these polypeptide growth factors bind to receptors on the cell surface and activate their tyrosine kinase activities. We have also observed a stimulation of RSVNEO mRNA in serum starved 3Y1 upon treatment with orthovanadate and this occurred in the presence of cycloheximide (data shown in the next chapter). Orthovanadate has been shown to inhibit tyrosine phosphatases and increase the level of phosphotyrosine in cells (Klarlund et al., 1985; Yonemoto et al., 1987). Finally, as shown in the next chapter, the tyrosine kinase, v-*src*, can relieve the requirement for serum and increase the amount of nuclear CCAAT box binding factor. All these lines of evidence point to the polypeptide growth factors in the serum which stimulate tyrosine kinases in the cell, e.g. IGF1, EGF, insulin, platelet derived growth factor (PDGF) etc., as being primarily responsible for the stimulatory action on the RSV LTR. This hypothesis is strengthened by the reported stimulation of the serum-response factor (SRF) that is responsible for the serum-induction of c-*fos*, not only by serum, but also by EGF and insulin (Prywes and Roeder, 1987; Fisch et al., 1987; Stumpo et al., 1988).

Are both the CCAAT boxes in the RSV LTR equally important in serum-induction? Are there other elements in the LTR that may play a role?

The difference in the activities of pm140CAT and ΔSM140CAT is of interest in addressing this issue. If, as has been proposed, a single DNA binding factor cannot provide a complete activation domain for the basic transcriptional machinery (Ondek et al., 1988), then it is conceivable that nuclear factors that bind to the SM fragment could interact with a CCAAT factor binding to the promoter proximal ("80") CCAAT box to provide a complete "activation domain" without requiring the presence of a CCAAT factor at the distal ("140") box. Such a complex could account for the relatively normal activity of pm140CAT and ΔPSCAT. In contrast, in the absence of the SM fragment, it becomes imperative that the LTR bind at least two CCAAT box factors to provide a complete activating domain for the basic transcriptional machinery, accounting for the low activity of ΔSM140CAT. Thus each CCAAT box can be seen as being the equivalent of an "enhanson", the loss of either of which can be partially compensated for by the SM fragment.

Another intriguing observation is the increased activity of dpm814CAT relative to pm80CAT, particularly because pm140CAT provides no evidence to suggest that the distal CCAAT box binds a repressor. Here too, the effects of similar mutations on the constructs that did not have the SM fragment was instructive. Since ΔSM814CAT did not have a higher level of activity compared to ΔSM80CAT, we can conclude that the apparent inhibitory effect of the distal ("140") CCAAT box in pm80CAT required the presence of an intact SM fragment. It can be envisaged that in pm80CAT, the factor(s) binding to the SM fragment could interact with the basic transcriptional machinery at the TATA box, but only by looping or twisting of the DNA at the "140" CCAAT box area and this is interfered with by a CCAAT factor bound to this segment of DNA.

Hence the apparent inhibitory effect of this CCAAT box shows up in the comparisons of pm80CAT and dpm814CAT. That the same sequence (and possibly, the same factor) can act as an activator in one context and repressor in another context adds to the diversity of transcriptional control that can be achieved with various permutations and combinations of a limited set of transcriptional factors and their binding sites.

Which CCAAT factor binds to the RSV LTR?

Many different CCAAT box factors have been identified by binding to CCAAT box oligos, and some have even been cloned (Dorn et al., 1987; Landschulz et al., 1988; Santoro et al., 1988; Paonessa et al., 1988; Gil et al., 1988; Chodosh et al, 1988a; Hatamochi et al., 1988). Our finding that a CCAAT factor belonging to the CTF/CP class binds to the RSV LTR over the two CCAAT boxes is in concordance with the reported ability of CBF obtained from rat liver to footprint on the RSV LTR, over both the "80" and "140" CCAAT boxes (Hatamochi et al., 1988; Maity et al., 1988). In our hands, the SM fragment could not compete for the factor binding to the RS fragment (data not shown) which would also conform with the results of Ryden et al.(1989) who found a different CCAAT factor, C/EBP, footprints over the enhancer in the SM fragment.

In parallel experiments, using nuclear extracts from HeLa cells, we found that a similar factor binds to the RS fragment through the two CCAAT boxes (data not shown). Methylation interference assay using this extract, revealed the contact points shown in Fig. 7. This pattern resembles that of CP2 (Chodosh et al., 1988a). Furthermore, the only bases outside the CCAAT sequence that are common between the oligos that competed for the RSV CCAAT factor and are different from the NF1 oligo (which does not bind the factor as effectively) are shaded, and it can be seen that the consensus sequence for CP2 contains the purine (R) at the 5th position 3' to the CCAAT, in contrast to the other consensus sequences. These two observations suggest that the factor

that we have identified may be related to CP2.

Are these findings with mammalian cells and the isolated LTR applicable to the intact retrovirus infecting chicken embryo fibroblasts?

As pointed out in the introduction to this chapter, there are reports in the literature which suggest that serum is important for the replication of the avian retrovirus in CEF. Two complicating factors must be borne in mind.

The first involves the changes imposed by the presence of a transforming oncogene, e.g. v-*src*. We have evidence suggesting that v-*src* can eliminate the serum requirement of the RSV LTR (Chapter 3). Bell et al. (1975) reported a drop in virus titer upon serum deprivation of a ts *src* infected culture of CEF held at the non-permissive temperature. The absence of any such drop upon serum deprivation of a wild type RSV infected culture when the medium was supplemented with $NaHCO_3$ (Leong et al., 1972) must have been due to the active v-*src* in the latter case. Further, Chen et al. (1974) showed that the levels of RNA and proteins from endogenous viruses in uninfected CEF was stimulated 4-5X upon serum-stimulation of serum-deprived cultures. It is interesting that though the LTR of one of the endogenous viruses, RAV-0, is smaller than that of RSV, and lacks a strong enhancer, it still contains a CCAAT box in the antisense strand at position -94 relative to the cap site (Hughes, 1982). Finally, in td107 infected CEF (these viruses have the same LTR as RSV, but do not contain an active v-*src*), we have found a 3-4x stimulation of steady state levels of viral RNA upon addition of serum to a serum-deprived culture and, as in mammalian cells, no protein synthesis was necessary for the serum stimulation (data not shown). In this light, the data presented by Humphries and Temin (1974), where they find no decrease in virus titer when ts NY68 RSV infected CEF at the NP temperature were made stationary by depleting the medium of "multiplication stimulating activity" (MSA), could be interpreted as suggest-

ing that the MSA deprived medium still contained serum factors that allowed viral transcription to continue.

An additional complication with CEF lies in the variation from embryo to embryo. We have found, by transfecting RSVCAT into various cultures of CEF, that some cultures are less dependent on serum than others. The reason for this variation is unclear, and could be due to growth-factor secreting cells that may be present in some primary cultures.

CHAPTER 3

In this chapter, we confirm the second part of the hypothesis outlined at the beginning of the last chapter, namely that the transformation of a cell by v-*src* relieves the serum requirement of transcription from the RSV LTR. We demonstrate that the effect is not due to the secretion of extracellular growth factors that substitute for serum and that the effect of v-*src* is mimicked by nonspecifically stimulating the level of phosphotyrosine in the cells by exposing them to an inhibitor of tyrosine phosphatases, sodium-orthovanadate. The serum inducible nuclear CCAAT binding factor that was shown to be important for the serum induction of transcription from the RSV LTR, is also shown to be induced by v-*src*, probably accounting for the serum independence of transcription from the RSV LTR in v-*src* transformed cells.

MATERIALS AND METHODS.

Cells and viruses.

3Y1 is an immortalized line of cells derived from rat fibroblasts.

tsNY 68 and tsNY 72 were prepared from molecular clones of the respective viruses as described (Nishizawa et al. 1985, Mayer et al. 1986). The viruses were pseudotyped by co-infecting chicken embryo fibroblasts (CEF) with either one of the ts viruses at 37^{o} C (permissive temperature for transformation of CEF) and a transformation-defective variant of Schmidt-Ruppin subgroup D (this subgroup can infect mammalian cells). The medium from the fully infected cultures, containing ts *src* virus pseudotyped as subgroup D, was used to infect 3Y1 in 10 % Fetal calf serum containing DEM (10% FCS

DEM). The cells were maintained at 32° C and in about ten days foci of transformed cells could be seen. These were isolated using cloning cylinders, grown up, tested for a temperature sensitive phenotype by growing them at 39° C, and recloned by dilution in multiwell tissue culture plates. Most of the experiments were done with a line called ts *src* 3Y1 derived from ts NY 68.

SR-3Y1 was derived from 3Y1 by transformation with wild type SR-RSV (Kawai et al., 1980). Most of my experiments were done with a clone derived from a colony in soft agar which maintains its transformed phenotype consistently in 5 % calf serum containing DEM (5% CS DEM).

ts *src* 3Y1 was transfected with 5 μg RSVNEO linearized at a unique *Bam* HI site that is outside the transcription unit of RSVNEO, and G418 resistant colonies were selected and pooled to prepare the cell line used in Fig. 11.

Immunoblotting.

ts *src* 3Y1 were maintained in 10 % FCS DEM at 39° C for 48 hrs. After changing the medium, the cells were shifted down to 34° C and harvested at the indicated time points. The procedure was as described in Hamaguchi et al. 1988. Briefly, the cells were washed in ice cold Tris glu, scraped off the plate, pelleted, and frozen in dry ice-ethanol. They were resuspended in Sol buffer (10 mM Tris HCl pH 7.4, 1 % SDS, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 1 mM PMSF) and heated to 100° C for 5 mins.. Protein concentration was assayed by the Bio-Rad dye binding assay, and 100 μg of proteins was analyzed per lane by SDS-7.5% polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically onto a nitrocellulose membrane with a transfer buffer (25 mM Tris glycine, pH 8.6, 20% methanol) and stained with anti-PTYR antibody (raised against bacterially expressed v-*abl* and purified with PTYR coupled

Sepharose) followed by ^{125}I labeled protein A (Amersham).

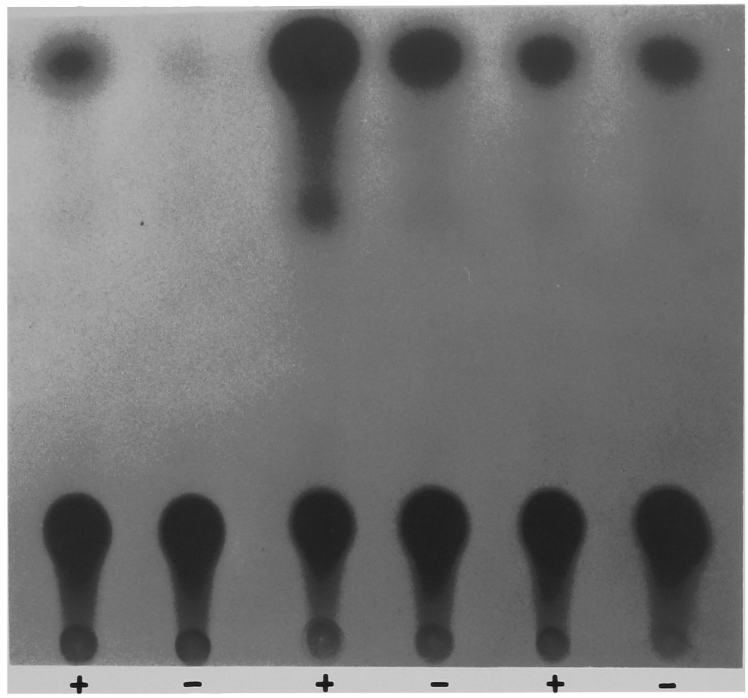
Northerns, S1 analysis, transfections, CAT assays, nuclear extracts and gel retardation assays.

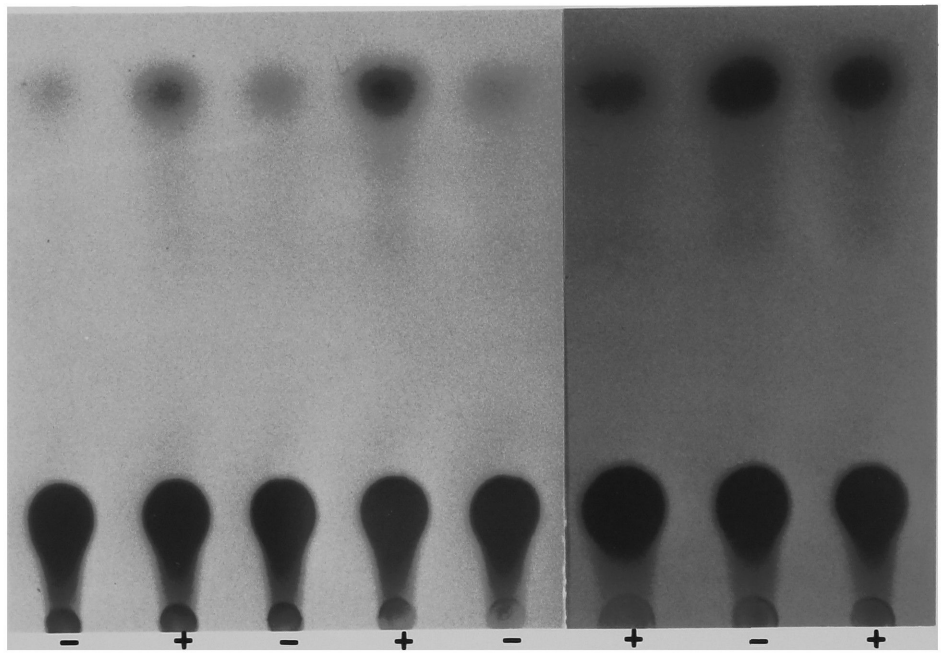
These were performed as described in the previous chapter.

FIGURE 8.

(A) (Upper picture) Serum-dependence of protein synthesis in 3Y1 cells and serum-independence of SR-3Y1 (SR) cells. "-": 48 hrs. in 0% DEM after transfection of RSVCAT. "+": 48 hrs. in 5% CS DEM after transfection of RSVCAT. sp: sparse culture of 3Y1; $5x10^5$ cells plated per 60 mm dish, 12 hrs. before transfection. d: dense culture of 3Y1 cells; these were plated at the same density as above, but were allowed to grow for 3 days, by which time they had reached saturation density.

(B) (Lower picture) Serum-independence of protein synthesis of SR-3Y1 cells is primarily an intra-cellular effect. For details of the experiment, see the text. "Transf": cells that received RSVCAT. "Mixed with": cells, not containing RSVCAT, which were mixed with the "Transf" cells, to test whether they could confer their own phenotype on the "Transf" cells. "U": untransformed 3Y1 cells. "T": transformed SR-3Y1 cells. "0": no cells. "-": cells in 0% DEM before harvesting. "+": cells in 5% CS DEM before harvesting.


+ − + − + −
3Y1 sp
3Y1 d
SR


− + − + − + − +
Transf U U U T
Mixed with U T 0 U

RESULTS.

Serum independence of protein synthesis of v-src transformed cells is primarily an intra-cellular effect.

Transfection of RSVCAT into 3Y1 cells, followed by 48 hrs. in the presence or absence of 5% calf serum resulted in CAT activity per plate which was 5-10 fold greater in the presence of serum than in its absence (Fig. 8A). In contrast, the CAT activity from v-*src* transformed 3Y1 (SR-3Y1) was not significantly different in the presence or absence of serum. The total protein content in the lysates from 3Y1 was greater by 2-5 fold in the presence of serum than in its absence, while in SR-3Y1 there was no difference in the protein contents. This implies that the effect of serum on CAT expression from the RSV LTR is a reflection of the serum dependence (or independence) of total protein synthesis in 3Y1 (or SR-3Y1).

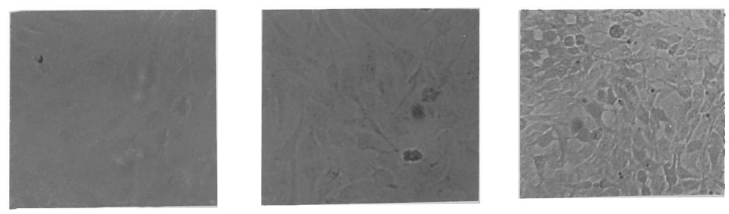
To determine whether the serum independence of protein synthesis in SR-3Y1 was due to the production of extra-cellular serum -substituting growth factors by the transformed cells, the crossfeeding experiment in Fig. 8B was done. RSVCAT was transfected into the indicated cell lines, which were then washed, trypsinized, pooled, counted and equal aliquots were mixed with equal numbers of the indicated cell lines, and replated in 5% CS DEM. After 12 hrs., the plates were washed with Tris-glu, and fed with either 0% DEM or 5% CS DEM. 30 hrs. later, the cells were harvested and the CAT activity per plate determined. It is clear from the results, that co-culturing 3Y1 with SR-3Y1 did not confer serum independence to the 3Y1 cells, nor did it inhibit the serum independence of SR-3Y1 cells. The conclusion is that the serum independence produced by v-*src* is primarily an intracellular effect.

FIGURE 9. The ts v-*src* 3Y1 cell-line.

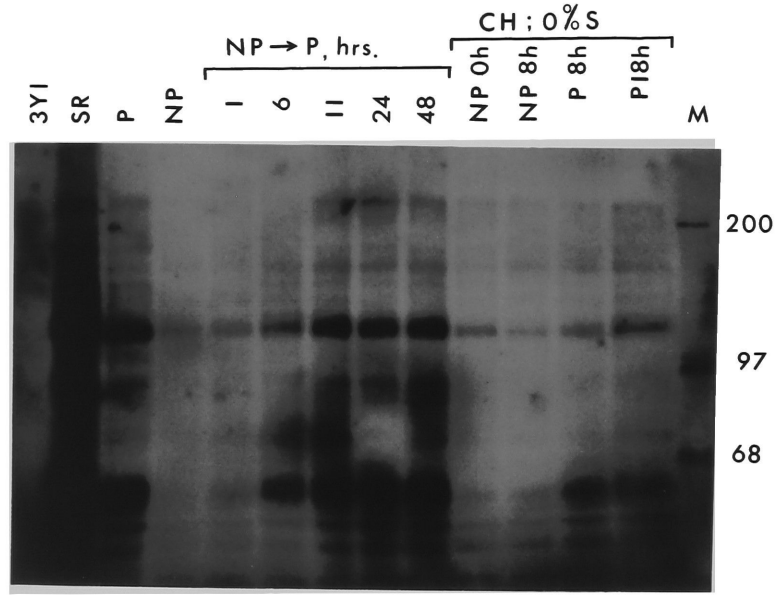
(A) Morphology of ts v-*src* 3Y1 at the NP temperature (39°C) for 72 hrs., and 14 hrs. (P-14) and 48 hrs. (P-48) after shift-down to P temperature (34°C).

(B) Western blot of proteins with antiphosphotyrosine (anti PTYR) antibody. The protein lysates were from 3Y1, SR-3Y1 (SR), ts v-*src* 3Y1 at 34°C (P), ts v-*src* 3Y1 at 39°C for 48 hrs. (NP), and ts v-*src* 3Y1 at the times indicated after shift-down from NP to P in 10% FCS DEM. The lanes under "CH; 0%S" were obtained from ts v-*src* 3Y1 at the NP temperature for 48 hrs., which were transferred to 0% DEM with 50 μg/ml cycloheximide and then kept at the temperatures indicated for the times shown before harvesting. M: molecular weight markers.

A


NP
P-14h
P-48h

B


3Y1
SR
P
NP
NP → P, hrs.
1
6
11
24
48
CH ; 0% S
NP 0h
NP 8h
P 8h
P18h
M
200
97
68

Temperature-sensitive v-*src* transformed 3Y1 line.

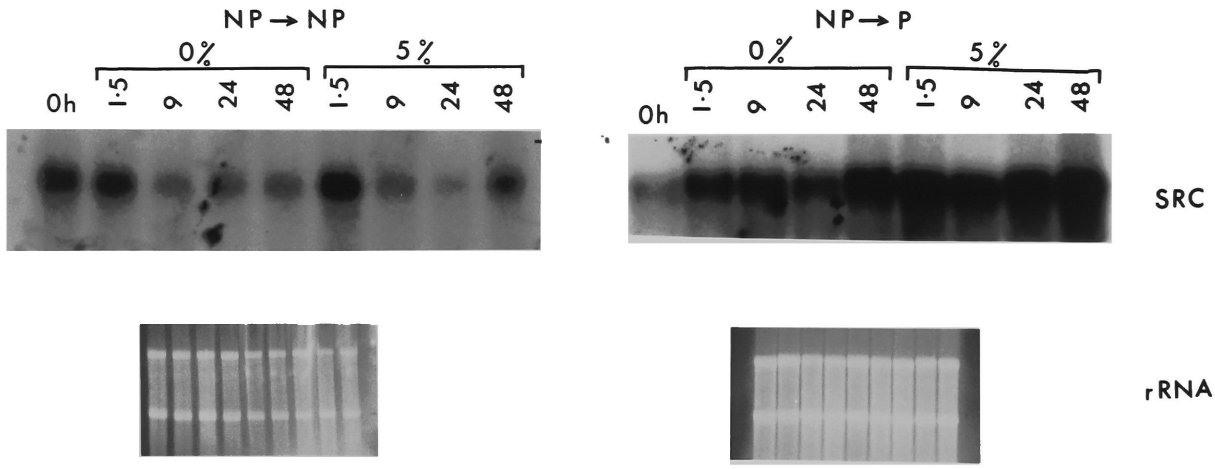
To test the effect of v-*src*, we created a ts v-*src* transformed 3Y1 cell line where we could switch the transforming activity of v-*src* on or off as needed. Fig. 9A shows that the cells demonstrate morphological transformation upon shift from the non-permissive (NP) temperature (39^{o}C) to the permissive (P) temperature (34^{o}C). Fig. 9B shows a Western blot analysis of protein lysates from the ts *src* transformed cells using anti-PTYR antibody. There is much more phosphotyrosine (PTYR) containing protein at the P temperature than at the NP temperature, though not as much as in the wild type v-*src* transformed SR-3Y1 (lane 2). Upon shift down to 34^{o} C there is a progressive increase in PTYR containing proteins. The 60 kd PTYR containing protein is likely to be pp60$^{v\text{-}src}$. When the shift down is carried out in 0% DEM in the presence of cycloheximide, an inhibitor of protein synthesis, there is still an observable increase in PTYR containing proteins (compare NP 8h with P 8h in the +CH lanes). This would be expected because the shift-down allows the pre-existing v-*src* protein to regain its activity and thereby increase the PTYR content in the cells without needing to synthesize new v-*src*.

v-*src* stimulates the levels of viral RNA in the presence and absence of serum.

The ts 3Y1 contains full length viral RNA and the spliced *env* and *src* RNAs, all of which initiate in the 5' LTR. Fig. 10 shows changes in the levels of the v-*src* RNA in these cells under various conditions. Though only the v-*src* mRNA is shown, the full length viral RNA and the *env* RNA behave in a similar manner. Cells grown at the NP temperature in 5% FCS DEM for 72 hrs., were washed with Tris-glu and fed with either 0% DEM or 5% FCS DEM and incubated for the indicated lengths of time at the NP temperature (39^{o}C) or the P temperature (34^{o} C). The v-*src* mRNA level dropped

FIGURE 10. Active v-*src* relieves the serum requirement of the RSV LTR and stimulates the levels of viral transcripts.

Northern blot of RSV RNA from the ts v-*src* 3Y1 cells under various conditions. Upper panels: v-*src* RNA. Lower panels: Ethidium bromide stained filter to show equal transfer of RNA in all lanes. "0h": ts v-*src* 3Y1 cells in 5% FCS DEM at 39°C (NP) for 72 hrs. Left panel: "0h" cells kept at 39°C after changing medium (0% DEM or 5% FCS DEM, as shown) and harvested at the times (in hrs.) indicated. Right panel: Same as left panel except plates were maintained at 34°C (P) after medium change. A lighter exposure of the right panel is shown to avoid loss of resolution in the 48 hr. lanes. The 0 hr. signals in both the panels are equal.


NP → NP
0%
5%
0h
1·5
9
24
48
1·5
9
24
48
NP → P
0%
5%
0h
1·5
9
24
48
1·5
9
24
48
SRC
rRNA

rapidly in 0% DEM and 5% FCS DEM at the NP temperature. However, once the temperature is lowered to activate the tyrosine-kinase activity of ts v-*src*, it can be seen (Fig. 10 Rt. hand panel) that active v-*src* relieves the serum requirement for maintenance of viral RNA levels (the continued persistence of v-*src* RNA in 0% DEM at the P temperature).

It is also of interest to note that the level of the viral RNA appears to be stimulated by the tyrosine kinase activity of v-*src*. This is evident from the progressive increase in the level of the v-*src* RNA with increasing time after shift-down, and the greater quantity of the RNA at the P temperature, compared to that at the NP temperature, at every time-point, both in the absence and presence of serum.

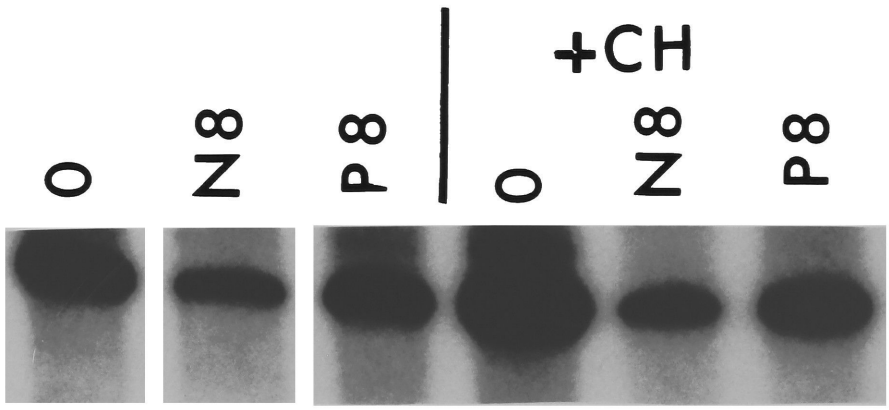
The drop in the viral RNA level at the NP temperature even in the presence of serum, might suggest that the effect of serum on the level of viral RNA, at the NP temperature, is short-lived. This is consistent with our observation that in untransformed 3Y1 cells, the addition of serum to a serum-deprived culture causes an elevation of LTR directed RNA levels that starts declining between 5 and 10 hrs after the stimulation (Fig. 1B).

Serum sparing effect of v-*src* on the levels of RNA transcribed from the RSV LTR is seen in the absence of new protein synthesis.

Since there is some increase in PTYR containing proteins in the ts *src* 3Y1 cells upon shift down in the presence of cycloheximide, in the absence of serum (Fig. 9), it was possible to test whether the serum-sparing effect of v-*src* was exerted without requiring new protein synthesis. The experiment is limited by the toxicity of cycloheximide in 0% DEM on the ts *src* 3Y1 at the NP temperature. Under these conditions, the cells remain viable upto about 12 hrs.. Using RSVNEO stably integrated into the ts *src* 3Y1, the level of transcripts initiating at the LTR was measured by an S1 Nuclease

FIGURE 11. Serum sparing effect of v-*src* is seen on the isolated RSV LTR and in the presence of cycloheximide.

S1 nuclease analysis of transcripts from RSVNEO in ts v-*src* 3Y1 cells containing RSVNEO stably integrated in the genome. The probe was RSVNEO cut and end-labelled at an unique *Nco* I site in the body of the NEO gene. Transcripts correctly initiated in the RSV LTR protect the 950 base long fragment shown. Left 3 lanes: cells grown in 10% FCS DEM at the NP temperature for 72 hrs. were transferred to 0% DEM (lane 0), and maintained at the NP temperature for 8 hrs. (N8) or the P temperature for 8hrs. (P8) before harvesting their total RNA for analysis. Right 3 lanes: same as above, except that cycloheximide at 10 μg/ml was added at the time of medium change.


+CH
O
N8
P8
O
N8
P8

assay (Fig. 11). Serum depletion of the cells at the NP temperature resulted in a discernible decline in the amount of NEO transcript by 8 hrs. (lane N8 vs lane 0). The serum sparing effect of the active v-*src* can be seen in a parallel culture held at the P temperature (P8). Addition of cycloheximide to 10 μg/ml to the plates at 0 hr. caused a two fold increase in the level of NEO transcripts (this is also seen in 3Y1, Fig. 1). However, the serum sparing effect of v-*src* was still evident even though new protein synthesis was blocked (compare lanes N8 and P8 under +CH).

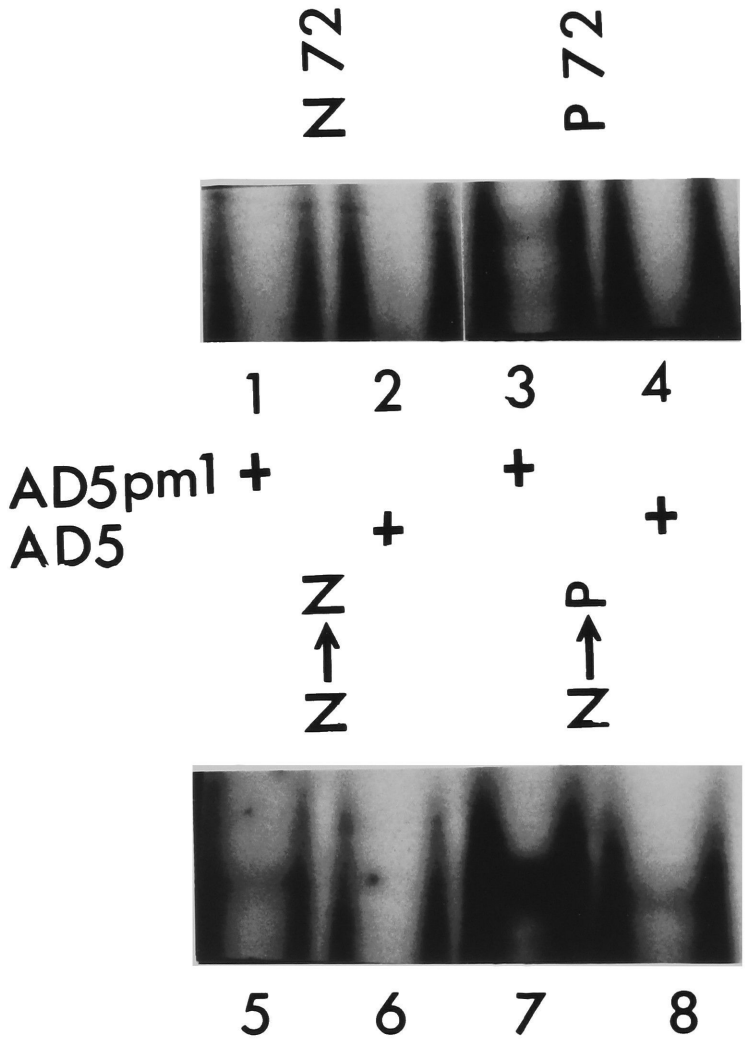
v-*src* induces nuclear CCAAT binding factor.

In the preceding chapter, it was shown that a CCAAT box binding factor belonging to the CTF/CP class, binds to the RSV LTR and mediates the serum responsiveness of the LTR. In view of the serum-sparing effect, and the stimulatory effect, of the tyrosine kinase activity of v-*src* on the level of RNA from the RSV LTR (Figs. 10 and 11), it was worthwhile to examine what effect v-*src* had on the nuclear CCAAT box binding factor that binds to the LTR.

Nuclear extracts were prepared from the ts *src* 3Y1 growing at 39^{o}C (NP) or 34^{o}C (P) for 72 hrs., in 10% FCS DEM, which was changed every 24 hrs.. The relative amounts of CCAAT box binding protein were measured using equal quantities of nuclear extract proteins in a gel retardation assay with the PS fragment (Fig. 2) of the RSV LTR (Fig. 12). Comparing lanes 1 and 3 leads to the conclusion that the active v-*src* at the P temperature induces the LTR CCAAT factor. In this experiment, both the bands seen in the gel shift assay (bands 1 and 2 : Fig. 5, Table 2) were visible and both were increased. Nuclear extracts from 3Y1 (not containing a v-*src* oncogene), grown at 39^{o} C and 34^{o} C under identical conditions, did not show any difference in the relative amounts of CCAAT binding factor (data not shown).

FIGURE 12. V-*src* stimulates the level of nuclear CCAAT factor.

Gel mobility shift assay using radio-labelled PS fragment (contains the distal CCAAT box of the LTR). Alternate reactions contain a plasmid carrying the intact PS fragment (pAD5) or a PS fragment with a mutation in the CCAAT box (pAD5pm1) as indicated. Only the specific DNA-protein complexes are shown (2 bands are seen in the experiment in the upper panel, while only the lower band is seen in the experiment in the lower panel). The nuclear extracts used in the upper panel are from ts v-*src* 3Y1 cells in 10% FCS DEM kept at 39°C for 72 hrs. (N 72, lanes 1 and 2), or at 34°C for 72 hrs. (P72, lanes 3 and 4). For the lower panel, the ts v-*src* 3Y1 cells grown in 10% FCS DEM at 39°C for 72 hrs. (N 72 conditions), were incubated for another 100 hrs. either at 39°C (N-->N, lanes 5 and 6) or at 34°C (N-->P, lanes 7 and 8) before preparing nuclear extracts.


N 72
P 72
1
2
3
4
AD5pm1 +
+
AD5
+
+
Z
Z
P
Z
5
6
7
8

Ts *src* 3Y1 growing at the NP temperature for 72 hrs. were either maintained at the NP temperature, or shifted down to the P temperature, and incubated for another 100 hrs., with the medium being changed every 36 hrs.. Nuclear extracts were made from these cells and equal quantities of protein were used for a gel shift assay. In this experiment, only the lower of the two bands (band 1) is seen, and it is induced strongly by the re-activated v-*src* at the P temperature (lane 7 compared to lane 5).

Orthovanadate stimulates transcription from the RSV LTR in serum starved cells.

v-*src* could be rendering the cells serum-independent by activating signal-transduction pathways that normally are activated only by extracellular growth factors present in the serum. An obvious mechanism would involve the tyrosine-kinase activity of v-*src* mimicking the tyrosine kinase activity of growth factor receptors interacting with their cognate ligands (e.g. insulin receptor, insulin like growth factor 1 (IGF1) receptor, EGF receptor, PDGF receptor etc.). If this was the case, then it could be predicted that non-specific elevation of PTYR content in untransformed 3Y1 would have a serum-mimetic effect on transcription from the RSV LTR.

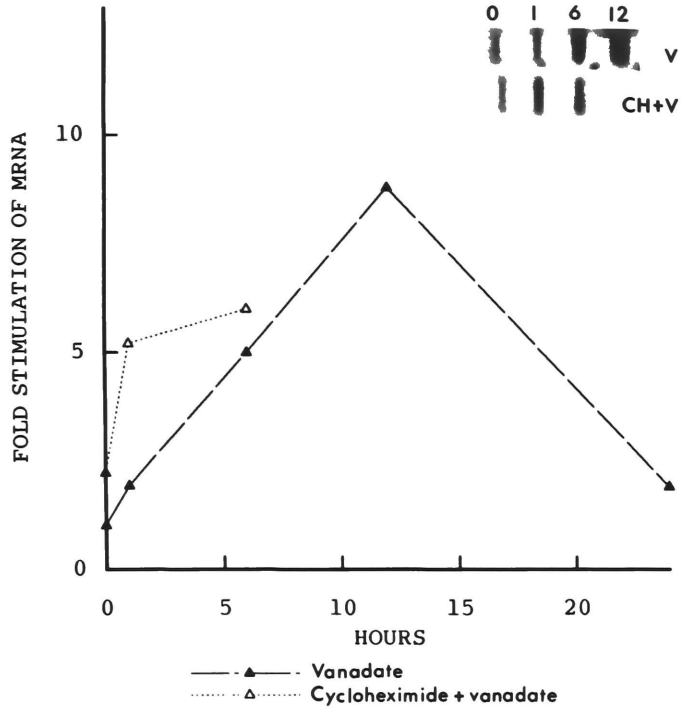
Fig. 13A shows the effect of adding sodium-orthovanadate to a serum-deprived culture of 3Y1 containing RSVNEO integrated into the cellular genome. The levels of NEO transcripts were followed by using equal quantities of total cellular RNA in a slot-blot-analysis and probing with a fragment from the NEO gene. A 10 fold stimulation of steady state level of NEO RNA was induced by 150 μM sodium-orthovanadate. Further, at least part of the stimulation persisted in the presence of an inhibitor of new protein synthesis (cycloheximide). Fig. 13B shows that most of the effect of orthovanadate on the RSVNEO transcript could be accounted for by a stimulation of transcription

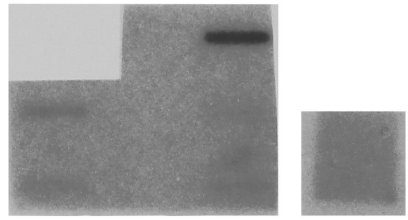
FIGURE 13. Orthovanadate mimics the effects of serum and v-*src* on the RSV LTR.

(A) Slot blot analysis of NEO transcripts from RSVNEO stably integrated into the genome of 3Y1. After serum deprivation (0% DEM) for 48 hrs. (0 hr.), the cells were exposed to 150 μM sodium orthovanadate and total RNA prepared at the times indicated. 3 μg RNA was used per slot (V). In a parallel experiment, cycloheximide was added to 50 μg/ml before adding vanadate. 1 μg RNA was used per slot (CH+V). All the signals were quantitated by densitometry of the autoradiograms, normalized to the signal from the 0 hr. (V) point and plotted.

(B) Nuclear run on analysis of the cell-line used above. Radiolabelled run-on transcripts were prepared from cells that were in 0% DEM for 48 hrs. (0) and from parallel cells that were exposed to 150 μM sodium orthovanadate for an additional 6 hrs. before harvesting. The blots contained the probes indicated (for details, see MATERIALS & METHODS of Chapter 2).

EFFECT OF ORTHOVANADATE





0 Vanadate

NEO

FOS TUB

28S PUC øX

(as measured by nuclear run-on assay) from the RSV LTR. The run-on assay also shows that the effect of orthovanadate on the RSV LTR was not part of a generalized, non-specific effect on transcription, because the promoters for c-*fos*, β tubulin and 28S ribosomal RNA genes were not stimulated by the same treatment.

DISCUSSION

How does v-*src* confer serum independence?

The results presented in Figs. 8A and 10 suggest that v-*src* transformed cells do not require serum to drive the expression of a gene by the RSV LTR. There have been other instances where the transformation of a cell by an oncogene confers the ability to grow independent of certain extracellular growth factors. v-*src* transformed fibroblasts require less serum (Hanafusa, 1977) and appear to not require EGF and Fibroblast growth factor (FGF) (Giguere and Gospodarowicz, 1983). v-*src* and related tyrosine kinase oncogenes also make chicken myeloid cells independent of chicken myelomonocytic growth factor (cMGF) (Adkins et al., 1984). v-*abl*, another tyrosine kinase oncogene, transforms fibroblasts and makes them independent of PDGF (Rees-Jones et al. 1989), erythroblasts of erythropoietin (Waneck et al. 1986), lymphoid cells of IL-3 (Mathey-Prevot et al. 1986) etc..

In some of the instances cited above, the factor-independent cells are found to secrete a protein into the extracellular medium accounting for the independence from exogenously added factor. Examples of this may be found in the secretion of transforming growth factor alpha and beta by v-*mos* transformed fibroblasts (Anzano et al., 1983), PDGF like molecules by a variety of transformed cells (Bowen-Pope et al., 1984), etc.. Upon transformation by v-*src*, myeloid cells produce cMGF (Adkins et al., 1984) and 3T3L1 murine fibroblasts produce a TGF beta like factor and a protease sensitive mitogen (Langer-Safer et al., 1985) that are detectable in the extracellular medium. In other instances, there does not appear to be an extracellular factor that can be detected in media conditioned by the transformed cells (Daley and Baltimore, 1988; Hariharan et al., 1988). The result presented in Fig. 8B supports the view that the serum-independence

of RSV LTR directed gene expression is primarily an intracellular effect.

The effect of v-*src* may involve the production of a growth factor inside the cell which stimulates its cognate receptor (intracellularly) and thus renders the cells serum-independent. Such may be the case with PDGF and the human osteosarcoma cell line U-2 OS CL 6 (Betsholtz et al., 1984) or IL-3 and *bcr-abl* transformed myeloid cell line FDC-P1 (Hariharan et al., 1988). However, in view of the serum sparing effect of v-*src* in the presence of cycloheximide (Fig. 11), we favor the hypothesis that v-*src* directly turns on the pathways that are normally stimulated by serum in 3Y1 cells.

What pathways could these be ? Since some of the effects of serum can be partially mimicked by insulin, IGF1 or EGF (preceding chapter, data not shown), all of which exert their actions through the tyrosine kinase activities of their receptors, the predominant signal transduced from serum (and activated by v-*src*) need not involve a single substrate but could involve an increase in the amount of PTYR on several key intracellular proteins activating multiple signal transduction pathways. The stimulation of transcription from the RSV LTR by orthovanadate, which inhibits phosphotyrosine phosphatases and nonspecifically increases the amount of PTYR on cellular proteins , supports this argument. An inhibitor of protein synthesis does not completely block the activation of transcription by vanadate, confirming that elevation of PTYR in the cells, whether by v-*src* or orthovanadate, directly sends the signal to the nucleus and argues against the signal being mediated by the synthesis of an intracellular growth factor.

How is the nuclear CCAAT box binding factor induced by v-*src*?

In the preceding chapter it was shown that the final effector of the signal transduced from serum to the RSV LTR in the nucleus could be a nuclear CCAAT box binding factor. This factor was induced by serum with a time course similar to the induction

of transcription from the RSV LTR, the induction of the factor occurred in the presence of cycloheximide (as was the induction of transcription), and mutations in the LTR that blocked the binding of the factor also blocked the serum induction of transcription from the LTR.

Fig. 12 shows that this same factor is induced by v-*src* in the presence of serum, providing a possible mechanism for the increased level of LTR directed mRNA synthesis seen in ts *src* 3Y1 at the permissive temperature (Fig. 10). Could an induction of the same CCAAT factor by v-*src* be responsible for the serum-sparing effect of the oncogene? We believe it could, because nuclear extracts made from 3Y1 cells show a rapid depletion of the CCAAT binding factor upon serum-deprivation while extracts from SR-3Y1 cells retain the CCAAT binding activity under identical conditions (data not shown).

The experiment to check if v-*src* could induce the CCAAT factor in the absence of protein synthesis turned out to be complicated by (a) the lower level of v-*src* kinase activity in the ts *src* 3Y1 cells compared to the wild type v-*src* transformed 3Y1 (SR-3Y1) (Fig. 9) and (b) the slow increase in tyrosine-kinase activity upon shift down to the permissive temperature (Fig. 9). Within the time period that the ts *src* 3Y1 at the NP temperature (the "no src" control) remains viable after the addition of cycloheximide, the net increase in kinase activity was barely sufficient to produce a 2 fold difference in the levels of RSV LTR directed transcripts (Fig. 11). A 2 fold (probably less) difference in the level of the nuclear CCAAT factor would not have been discernible in the nuclear extracts that were prepared. However, in view of the ability of serum to induce the CCAAT factor in the presence of cycloheximide (previous chapter), and the ability of v-*src* (Fig. 11) and vanadate (Fig. 13) to exert their serum-mimetic effects on the RSV LTR in the presence of cycloheximide, it appears likely that the tyrosine-kinase activity

of v-*src* would be able to induce nuclear CCAAT factor without needing new protein synthesis.

This does not rule out an additional effect of v-*src* on the CCAAT factor mediated through the synthesis of new proteins. In fact, the slow and progressive increase of LTR driven gene expression upon shifting the ts *src* 3Y1 cells to the permissive temperature, suggests that at least some of the effect of v-*src* on the LTR is not a primary effect. One could imagine a scenario where the initial increase in nuclear CCAAT factor induced by post-translational mechanisms leads to changes in transcription of genes coding for various components on the signal transduction pathway. If protein synthesis is allowed to proceed, then these new transcripts would be translated, resulting in more profound changes in cellular physiology. One obvious candidate for such a protein would be v-*src* itself. In fact, the slow and progressive increase of the $pp60^{v\text{-}src}$ band upon shift-down (Fig. 9) supports this view. A second candidate for such a protein would be the CCAAT factor itself. Gel retardation analysis of nuclear extracts prepared at various times after shifting ts *src* 3Y1 cells to the P temperature, shows a discernible difference in the levels of CCAAT factor per μg of nuclear protein between the NP and P cells only after 24 - 36 hrs. (data not shown), and this slowly increases to the difference seen at 100 hrs. after shift down (Fig. 12), implicating a slow mechanism in the induction of the CCAAT factor. Could this involve induction of the gene for the CCAAT factor itself? This hypothesis can be tested easily using cloned CCAAT factor (CTF or NF1) (Santoro et al., 1988; Paonessa et al., 1988; Gil et al., 1988) as a probe on the Northern blots of Fig. 10.

What could be the mechanisms by which v-*src* induces nuclear CCAAT factor? The models suggested for serum induction of the CCAAT factor (in the preceding chapter) are equally applicable for induction by v-*src*. The common mechanism between

the two pathways, as also for the induction by orthovanadate, would be an increase in the PTYR content of critical cellular proteins.

Are the effects of v-*src* on the RSV LTR in rat cells relevant to RSV infection of CEF ?

In tsNY68 or tsNY72 infected culture of CEF, when total cellular RNA was harvested at various times after shift-down to the permissive temperature, and the RNA examined by Northern blot analysis, we found a 3-4X increase in the levels of all the viral RNAs within 0.5-1 hr. after shift-down (data not shown). This corresponds well to the rate of increase of PTYR containing proteins after shift-down (Hamaguchi et al., 1988). However, the elevation in the level of viral RNA did not persist, having decreased to levels below that found in parallel cultures held at the NP temperature by 6-12hrs.. This is in contrast to the result with the ts *src* 3Y1 (Fig. 10) and could be due to detrimental effects of increased viral replication and spread on the CEF. It is of interest that a similar feature of induction followed by return to NP levels is seen with several other v-*src* induced messages in CEF (Simmons et al., 1989).

The effect of orthovanadate on the level of viral RNA in serum deprived CEF, infected with td107 (a transformation defective variant of RSV), is identical to the effect on RSVNEO in 3Y1 shown in Fig. 13 (data not shown).

Finally, if v-*src* had a stimulatory effect on expression from an avian retroviral LTR in CEF, particularly a serum-sparing effect, then it would resolve the contradiction in the literature between the results of Leong et al. (1972), who found no drop in the levels of virus titer upon serum deprivation of cells that are already transformed by SR-RSV (provided that the pH of the medium was maintained by adding $NaHCO_3$) and those of Bell et al. (1975), who found a 100 fold drop in titer of infectious virus upon

serum deprivation of a ts *src* transformed culture of CEF held at the non-permissive temperature.

CHAPTER-4.

It is progressively becoming evident that the transcriptional machinery of eukaryotes has been preserved such that there are many components in common between species as divergent as Saccharomyces cerevisiae and Homo sapiens. The steroid receptors and their transcriptional response elements from humans work in yeast (Schena and Yamamoto, 1988; Metzger et al., 1988). The bacterial repressors and operators work in yeast and in animal cells (Brent and Ptashne, 1984; Hu and Davidson, 1987; Brown et al., 1987; Figge et al., 1988). Several yeast transcriptional factors appear to be homologous to factors in animal cells, e.g. GCN4 and AP1 (Struhl, 1987; Harshman et al., 1988), HAP2/HAP3 and the CCAAT box binding factors of HELA cells (Chodosh et al., 1988a, 1988b) and yeast PRTF/GRM and the c-fos SRF (Norman et al., 1988). GAL4, a yeast transcription factor works in animal cells (Kakidani and Ptashne, 1988; Webster et al., 1988). Even biochemically purified TATA box binding factor from yeast directs transcription in vitro using a HELA cell transcription extract (Buratowski et al., 1988; Cavallini et al., 1988).

In this light, we examined whether the RSV LTR directs transcription in Saccharomyces, on the assumption that if it did, it would be an ideal system for cloning transcription factors that interact with the LTR in yeast, taking advantage of the genetic approaches available in yeast. Further, yeast would provide an ideal system for delineating various factors that interact with each other and thereby regulate the transcription from the LTR. Finally, once yeast genes have been identified that are responsible for regulating transcription from the LTR, it should be possible to clone the homologues from animal cells by their ability to complement the mutations in the yeast genes.

We find that the LTR directs accurate initiation of transcription in yeast, probably uses the same TATA box that is used in mammalian cells, and also uses the same CCAAT boxes as an "upstream activating sequence" (UAS). Furthermore, mutations affecting these essential sequences gave a phenotype to the yeast carrying them, so that it will be possible to devise selection strategies for isolating mutations in yeast transcription factors, and possibly clone the genes for yeast and mammalian transcription factors and do structure function analysis on them. We also tested whether v-*src* stimulates the level of transcripts from the RSV LTR in yeast, and the answer currently is a tentative "yes".

MATERIALS AND METHODS.

Plasmids.

RSVCAT, ΔSMCAT, ΔRSCAT, ΔPSCAT, pm80CAT and dpm814CAT have been described. ΔHRCAT was made by cutting 51BgCAT (described in Chapter 2) at the unique *Bgl* II and *Hind* III sites, blunting with Klenow, and ligating with T4 DNA ligase. Parental forms were selected against by cutting once again with the same restriction enzymes, transforming E. coli and screening some of the colonies for a plasmid with the required deletion. SV_2CAT (Gorman et al., 1982a) has the SV40 enhancer and early promoter directing expression of the bacterial CAT gene.

All the plasmids mentioned above (except 51BgCAT) were each cut at an unique *Bam* HI site which is outside the CAT transcription unit, treated with Bacterial alkaline phosphatase, and ligated with a 1.9 kb *Bam* HI fragment encoding the HIS3 gene of Saccharomyces cerevisiae. Standard procedures (Maniatis et al. 1982) were used to screen for plasmids having one 1.9 kb fragment inserted into the corresponding CAT plasmid. The CAT plasmids with the HIS3 auxotrophic marker were named XCAT+HIS (e.g. RSVCAT+HIS) if the directions of transcription of HIS3 and CAT were the same and XCAT-HIS if they were opposite to each other.

pFL is derived from pBR322 and contains the "autonomous replication sequence" (ARS) of a 2 μm plasmid inserted into the *Eco* RI site, and the yeast URA3 gene in the *Hind* III site. The 4.7 kb *Bam* HI to *Bgl* I fragment of pFL is ligated to the 3.5 kb *Bam* HI to *Bgl* I fragment of RSVCAT to give FRSVCAT. This plasmid has a ColE1 origin of replication, an ampicillin resistance gene, the RSV LTR directing transcription of the CAT gene, the URA3 gene, and the origin of replication of a 2 μm episome from yeast.

YEP 51 (Broach et al. 1983) contains the yeast LEU2 gene under control of its own promoter, a GAL 10 promoter, the origin of replication of a yeast 2 μm plasmid, a bacterial ampicillin resistance gene and a bacterial ColE1 origin of replication. There are 3 *Eco* RI sites, one in the LEU2 gene and two flanking the 2 μm origin of replication. Y-vsrc (Kornbluth et al. 1987) contains the v-*src* gene integrated into YEP 51 such that it is under the control of the GAL 10 promoter. To make the integrating plasmids IP 51 and I-vsrc, YEP 51 and Y-vsrc were each partially digested with *Eco* RI, ligated, parental forms selected against using *Xba* I (which cuts once in the *Eco* RI fragment encoding the 2 μm origin of replication), and the reaction products used to transform E. coli RR1 (leu B^-) to Ampicillin resistance and LEU^+. The colonies growing on minimal agar, supplemented with proline and containing ampicillin, all contained the desired plasmids with the 2 μm ARS deleted out.

Strains and media.

The strains of S. cerevisiae used were W303-1A (haploid, MATa, ade 2-1, ura 3-1, leu 2-3,112, his 3-11,15, trp 1-1, can 1-100), BWG1-7A (MATa, leu 2-3,112, his 4-519, ade 1-100, ura 3-52) and its derivatives hap 2-1 (Guarante, 1984) and Δhap3 (Olesen et al., 1987; Hahn et al., 1988). The yeast were grown in synthetic or YEP media containing 2% glucose, 3% lactate, 2% galactose or 2% raffinose.

Transformation of yeast, RNA and DNA analysis.

These were all done using standard protocols (Sherman et al. 1986 and Maniatis et al., 1982). For establishing the lines of yeast containing RSVCAT and its derivatives, we started with W303-1A. These were transformed with the CAT+HIS plasmids that had been linearized at a *Xho* I site in the HIS3 gene, using the Lithium chloride treatment

protocol, and selected on minimal medium (SD) supplemented with adenine, uracil, leucine and tryptophan (his^-). A few of the colonies were grown up in SD (his^-) followed by at least 15 -20 generations of growth in YPD (no selection). The yeast were then plated at the appropriate dilution on YPD and replica plated onto SD (his^-). If all the colonies that grew up on YPD were also HIS^+, the CAT+HIS plasmids were considered to be integrated into the yeast genome.

To ensure that the yeast we studied had only one copy of the CAT gene per haploid genome, DNA was prepared from them, digested with *Eco* RI and *Bam* HI, and a Southern analysis was performed using nick translated RSVCAT and pTRP1 (contains the yeast TRP1 gene cloned into pUC 18).

Total RNA was prepared from the yeast by vortexing them in the presence of glass beads, phenol and LETS buffer (0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris HCl pH 7.4, 0.2% SDS), followed by phenol extraction and chloroform extraction of the aqueous layer. S1 analysis and Northerns were performed as described in Chapter 2.

Primer extension was done using 50 μg of total RNA in 10 μl vol containing 50 mM Tris HCl pH 7.5, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM of each of the four dNTPs and 20 ng of CAT24 oligo (5'CTCCATTTTAGCTTCCTTAGCTCC3') labeled at the 5' end using T4 polynucleotide kinase and $\gamma^{32}P$ ATP. The mixture was heated to 85^o C for 5', cooled slowly to room temperature (25^o C), 200 units of MoMuLV reverse transcriptase was added, and the primer extension was allowed to proceed for 60 mins. at 37^o C. The sequencing ladder was created by double stranded plasmid sequencing using RSVCAT as the template and the labeled CAT24 oligo as the primer.

CAT assays.

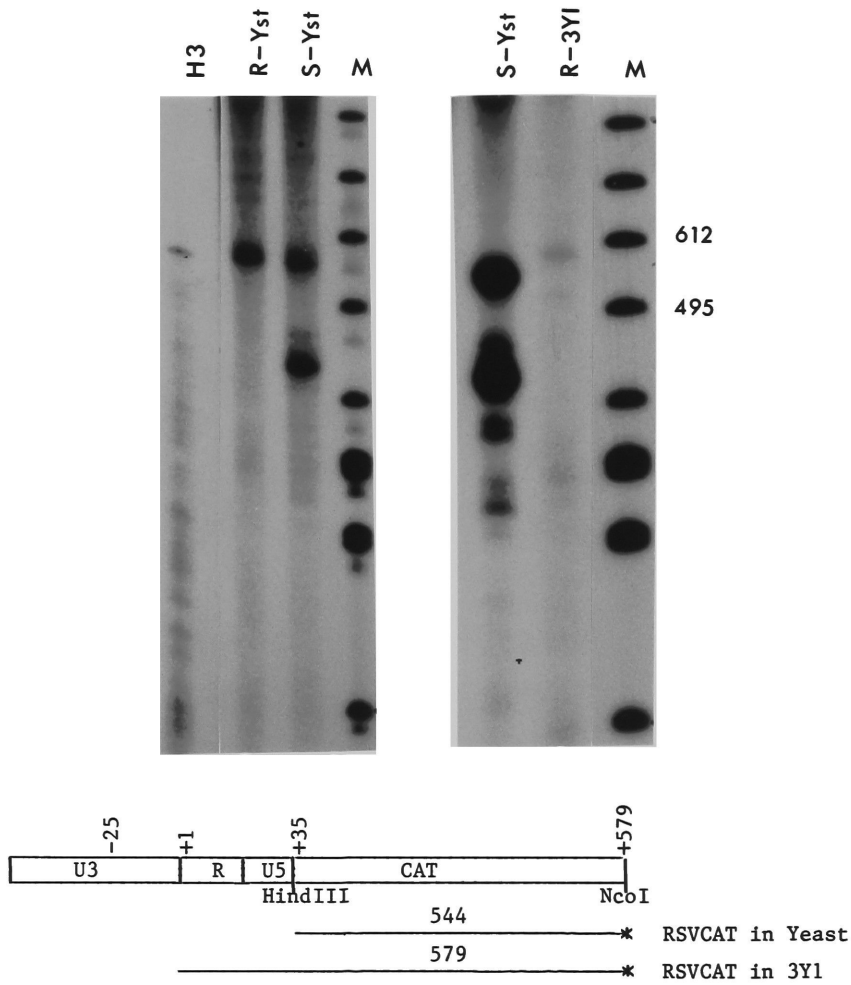
Overnight cultures of the various strains in YPD were diluted 1:5 in fresh YPD and incubation at 30°C continued for 3 hrs.. The yeast were then harvested by centrifugation, washed with water, suspended in 200 μl of 0.25 M Tris HCl pH 7.8, and broken by vortexing with glass beads for 15 mins. at 4°C. The aqueous layer was collected after spinning for 5 mins. in the microfuge at 4°C, and any particulate matter removed by a second spin of 10 mins.. The protein content in each lysate was measured using the Bio-Rad dye binding assay. 1 μg of total protein was used for the CAT assay using 0.25 μCi of ^{14}C Chloramphenicol (NEN, 60 mCi/mmol) as described before (Chapter 2). The reaction was performed at 37°C for 10 mins..

Immunoprecipitation kinase assays.

150 ml of an overnight culture of each strain of yeast was grown up in 0.67% yeast nitrogen base, 2% w/v raffinose and supplemented with adenine, tryptophan and uracil. The cultures were pelleted and resuspended in the same volume of a similar medium containing 2% w/v galactose instead of raffinose. Incubation was continued for 4 hrs at 30°C before the cells were harvested and lysed in RIPA buffer (10 mM Tris HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and 1% (v/v) Trasylol) as described in Kornbluth et al. (1987). Immunoprecipitation with mAb 327 (Lipsich et al. 1983) and in vitro kinase assay were performed as described (Kornbluth et al., 1987).

FIGURE 14.

S1 nuclease analysis of RNA from yeast carrying one copy of RSVCAT (R-Yst) or SV_2CAT (S-Yst) and from 3Y1 cells transiently transfected with RSVCAT (R-3Y1). "H3": fragment produced by cutting the probe with *Hind* III. The probe was RSVCAT, cut and labelled at an unique *Nco* I site in the CAT gene. The sizes of the fragments protected are correlated with a schematic of RSVCAT at the bottom of the figure. "U3, R and U5" are parts of the RSV LTR. SV_2CAT diverges in sequence from RSVCAT upstream of the *Hind* III site and so all transcripts present in "S-Yst" that initiate upstream from this point protect the probe only upto the *Hind* III site. "M": size markers obtained by *Hinc* II digestion of ϕX DNA.


H3
R-Yst
S-Yst
M
S-Yst
R-3Yl
M
612
495
-25
+1
+35
+579
U3
R
U5
CAT
HindIII
NcoI
544
RSVCAT in Yeast
579
RSVCAT in 3Yl

RESULTS.

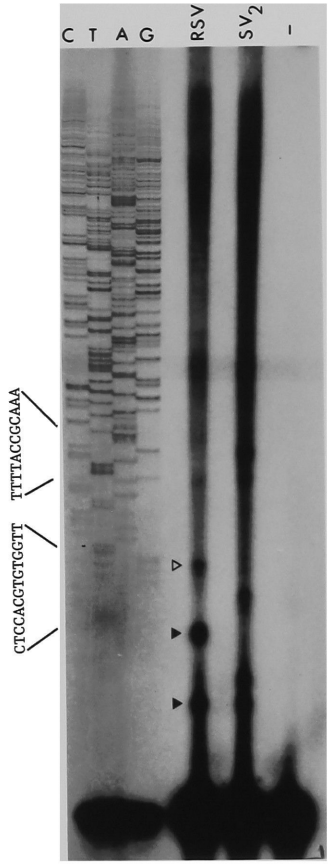
The RSV LTR directs accurate initiation of transcription in S. cerevisiae about 60 bases downstream from the TATA box.

Total cellular RNA was prepared from yeast containing one copy of RSVCAT integrated at the HIS3 locus in the genome. S1 nuclease analysis using RSVCAT cut and end-labeled at the *Nco* I site as the probe (Fig. 14), suggested specific initiation of transcripts near the *Hind* III site which is 544 base upstream from the *Nco* I site (compare the size of the protected fragment in the R-Yst lane with the fragment generated by cleaving the probe with *Hind* III). This shows that the RSV LTR in yeast may be directing initiation of transcription about 60 bases downstream from the TATA box.

RNA from yeast containing SV_2CAT was used as a control for specificity of the S1 assay and showed a different pattern of protection (S-Yst). The smaller protected fragment was probably produced by RNA initiating at a specific site in the body of the CAT gene. The larger protected fragment is an artifact resulting from the divergence in sequence of the SV_2CAT plasmid from the RSVCAT probe upstream from the *Hind* III site. Consequently, transcripts initiating upstream of this *Hind* III site protect the *Nco* I - *Hind* III (544 base) fragment. This larger protected fragment in the S-Yst lane therefore marks the position of the *Hind* III site. Referring to this size marker in the right hand panel of Fig. 14 clearly indicates that in contrast to the situation in yeast, RNA derived from transient transfection of RSVCAT into mammalian cells (3Y1) initiates at a site about 35 bases upstream from the *Hind* III site. This is the authentic initiation (+1) site of the RSV LTR in mammalian and chicken cells (about 25 bases from the TATA box).

FIGURE 15.

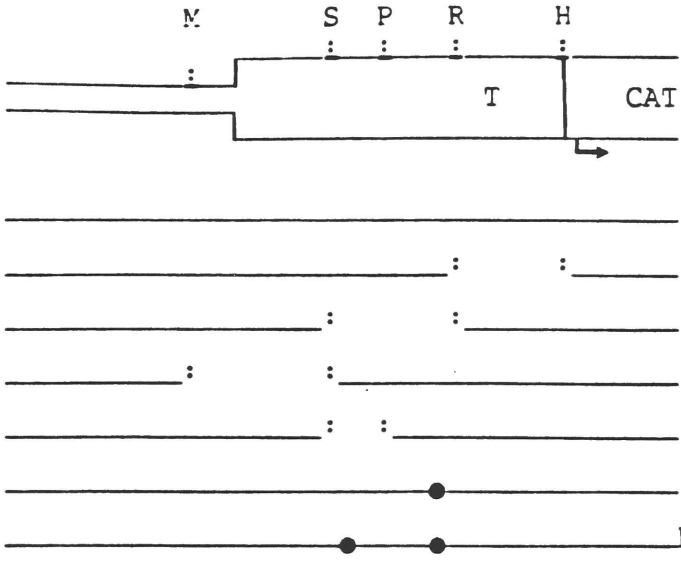
Primer extension analysis of RNA from yeast containing one copy of RSVCAT (RSV) or SV_2CAT (SV_2). "-": no RNA added to the reaction. The primer was the CAT 24 oligo described in the MATERIALS & METHODS and the 3' extent of it is indicated on the sequence at the bottom of the figure. A sequencing ladder was created using the same oligo on RSVCAT and was run alongside the primer-extension products. The sequencing lanes are presented such that reading from bottom to top one reads the sequence of the sense strand of RSVCAT from 3' to 5'. The closed and open triangles mark the specific extension products seen with the yeast containing RSVCAT and correspond to the major and minor initiation sites respectively. On the sequence at the bottom, the TATA box is underlined, and the major and minor transcription start sites indicated by closed and open boxes, respectively. The numbers are relative to the transcription start site in animal cells (+1). "H3": *Hind* III site. "U3, R, U5": parts of the RSV LTR.


C T A G
RSV
SV2
-
TTTTACCGCAAA
CTCCACGTGTGGTT


-30
+1
+25
+38
+44
TATTTAAGTGCCTAGCTCGATACAATAAACGCCATTTTACCATTCACCACATTGGTGTGCACCTCAAGCTTGCGAGATTTTCAGGAGCTAA
U3
R
U5
H3
primer

FIGURE 16.

Amount of CAT expressed from various mutant derivatives of RSVCAT present in single copy in yeast. A schematic of the RSVCAT plasmid is given at the top, and the major transcription start site indicated with an arrow. H: *Hind* III, R: *Eco* RI, P: *Pvu* I, S: *Sph* I, M: *Mst* II. T: TATA box. HRCAT, RSCAT, SMCAT, PSCAT are the corresponding Δ plasmids. Gaps bounded by restriction enzyme sites indicate the extents of the deletions, and the dots mark the positions of the point-mutations. For the exact sequences of the point mutations, see Fig. 2. The CAT activities are given in the units shown, along with the standard errors, and the number of independent measurements.



	% Chloramphenicol acetylated per ug protein per minute
RSVCAT	4.2 ± 0.4 (6)
HRCAT	0.03± 0.01(3)
RSCAT	1.75± 0.5 (6)
SMCAT	7.4 ± 0.3 (4)
PSCAT	5.45± 1.0 (4)
pm80CAT	4.7 ± 0.4 (3)
pm814CAT	2.4 ± 0.15(3)

FIGURE 16

To confirm this finding, a primer extension analysis was done using the CAT24 oligo as a primer (Fig. 15). It can be seen that the transcripts from the RSV LTR in yeast initiate at two major sites near the *Hind* III recognition sequence and one minor site further upstream. Clearly, no initiation can be seen at the beginning of R (the +1 for higher eukaryotic cells). Again RNA from yeast containing SV_2CAT was used as a control for the specificity of the RSV LTR.

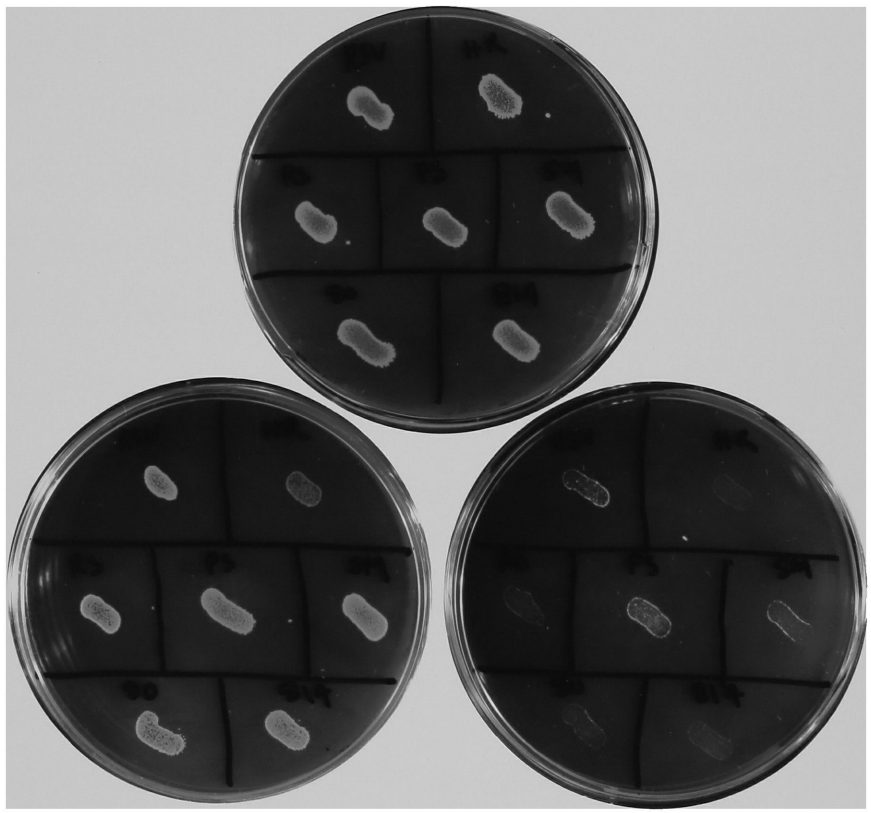
The TATA box and the CCAAT boxes of the RSV LTR are required for expression from the LTR in yeast.

The structure of the LTR was shown in Fig. 2 along with some of the point-mutations made in the RS fragment. To determine which parts of the LTR had demonstrable functions in directing expression from it in S. cerevisiae, The plasmids indicated in Fig. 16 were each linked to a HIS3 gene and introduced into yeast. Results of CAT assays performed with extracts from the yeast containing the XCAT+HIS plasmids are shown. The deletion in ΔHRCAT appears to remove a very critical function of the LTR. Since the major initiation site in yeast (Fig. 15) is left intact in this deletion, one could presume that the critical element removed is the TATA box. Examination of the sequence removed reveals only the TATA box shown in Fig. 15 as resembling the canonical TATA box sequences. This is the same TATA box used for transcription from the RSV LTR in animal cells (Gilmartin and Parsons, 1983).

ΔRSCAT and dpm814CAT had half the activity of the intact RSVCAT. One conclusion that may be drawn is that the two CCAAT boxes in the RS fragment act as "upstream activating sequences" (UAS). The lack of a significant decrease in CAT activity with pm80CAT and ΔPSCAT (which deletes the distal, "140", CCAAT box) indicates that both the CCAAT sequences are functional in YPD, and either of them

FIGURE 17.

Ability of RSVCAT and its derivatives to confer resistance to chloramphenicol. Yeast containing one copy of each of the plasmids indicated (XCAT+HIS) were grown on synthetic medium without histidine and with lactate as the only carbon source. They were then spotted on agar plates with the same medium but containing chloramphenicol at 0 mg/ml (top plate), 1 mg/ml (bottom left) and 2 mg/ml (bottom right). On each plate, the yeast containing the CAT plasmids were spotted in the following arrangement from left to right: in the top row, RSVCAT, ΔHRCAT; in the middle row, ΔRSCAT, ΔPSCAT, ΔSMCAT; in the bottom row, pm80CAT, dpm814CAT.



alone can function effectively as an UAS.

The deletions in the LTR confer a distinct phenotype to the yeast.

It has been shown that in S. cerevisiae growing on a non-fermentable carbon source (e.g. lactate), chloramphenicol is toxic to the yeast, and that expression of chloramphenicol acetyl transferase (CAT) relieves this toxicity (Cohen et al., 1980). Fig. 17 demonstrates that RSVCAT present in a single copy in the genome allows the yeast to grow on lactate at concentrations of chloramphenicol upto 2 mg/ml. As would be predicted, the ΔHR deletion construct is unable to support growth at 1 mg/ml chloramphenicol, while the ΔRSCAT, pm80CAT and dpm814CAT are able to confer resistance to 1 mg/ml but not to 2 mg/ml chloramphenicol. The fact that the ΔPSCAT could confer resistance upto 2 mg/ml of chloramphenicol while pm80CAT could not, implies that under these growth conditions, the proximal CCAAT box alone is critical for the UAS function.

The products of HAP2 and HAP3 genes are not involved in transcription from the RSV LTR in yeast.

Since the CCAAT boxes in the RSV LTR have been shown to bind specific transcription factors in rat cells (chapter 2), and since it has been demonstrated that the gene products of HAP2 and HAP3 in yeast bind to CCAAT like sequences and are activators of transcription from several yeast promoters (Chodosh et al., 1988b), it was possible that the CCAAT sequences in the LTR were binding the HAP2 and HAP3 gene products. This possibility appeared even more likely upon observing the sequence similarity of the UAS2UP1-CYC1 CCAAT box and those of the RSV LTR (Fig. 18). However, two observations make this unlikely.

The genes that are activated by HAP2/HAP3 are induced when grown on a non-fermentable carbon source (e.g. lactate) as opposed to glucose. For instance, beta-

FIGURE 18.

Sequences of CCAAT elements that play a role in transcription in yeast. RSV80: the promoter proximal CCAAT box in the RSV LTR. RSV140: the distal CCAAT box in the RSV LTR. UAS2CYC1: actually the UAS2UP1-CYC1 upstream of the CYC1 promoter (Chodosh et al, 1988b). TY1 II: Block II sequence in the Ty 1 element (Company and Errede, 1988). The positions of the mutations in a Block II oligomer that inactivated its "UAS" activity and its ability to bind a sequence specific DNA binding protein are indicated by "^" below the sequence.

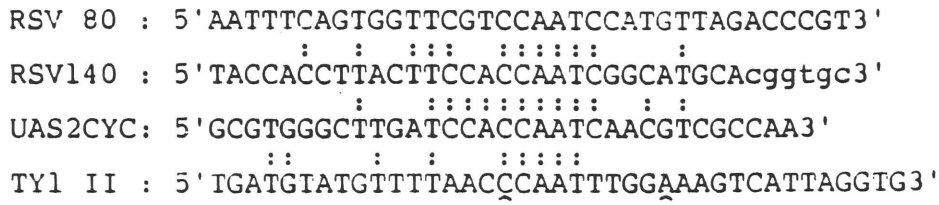

RSV 80 : 5'AATTTCAGTGGTTCGTCCAATCCATGTTAGACCCGT3'
RSV140 : 5'TACCACCTTACTTCCACCAATCGGCATGCAcggtgc3'
UAS2CYC: 5'GCGTGGGCTTGATCCACCAATCAACGTCGCCAA3'
TY1 II : 5'TGATGTATGTTTTAACCCAATTTGGAAAGTCATTAGGTG3'

FIGURE 18

TABLE 3

1. Plasmid	2. Growth Conditions.	3. % Chloramphenicol acetylated per µg protein per minute.	
		Glucose	Lactate
RSVCAT-HIS	O.N. culture in minimal medium.	10	0.9
RSVCAT-HIS	O.N. culture in minimal medium.	2.5	0.3
RSVCAT+HIS	O.N. culture in YP diluted 1:5 grown for 3 hrs.	4.0	0.02

1. Yeast containing one copy of the indicated plasmid integrated into the genome were used.
2. O.N. : overnight saturated culture.
3. The activity is shown below the carbon source used in the growth medium.

TABLE 4

1. Plasmids	2,3. Growth Conditions.		4. CAT activity in yeast with v-src / CAT activity in yeast with vector alone.
RSVCAT-HIS + Y-vsrc or YEP 51	S. Lactate. O.N.		1.6 0.8 0.3
	S. Glucose. O.N.		1.6 1.8 0.6 2.0
	S. Raffinose. O.N.		2.7 0.5
RSVCAT+HIS + I-vsrc or IP 51	Y. Glu O.N.	1:5 Y.Glu	0.42
		1:1 Y.Glu.	1.04
		1:5 Y.Gal.	1.11
		1:1 Y.Gal.	0.84
	S. Glu O.N.	1:1 S.Glu.	1.0
		1:1 S.Gal.	2.3

1. Yeast containing one copy of the CAT plasmid were used. The yeast also contained either one of the multicopy episomal Y plasmids shown or one of the integrated I plasmids.

2. S : Synthetic medium with appropiate additions to maintain selection for the plasmids. Y : YEP medium. Glu : glucose. Gal : galactose. O.N. : overnight saturated cultures (at least 16 hrs.) in the indicated medium.

3. In the bottom half of the table, the O.N. cultures indicated are pelleted, washed and resuspended in the media shown at the dilutions shown and allowed to incubate with shaking for 3 hrs. before harvesting.

4. The ratios of the % chloramphenicol acetylated per minute per microgram of protein extract from yeast containing Y-vsrc (or I-vsrc) to that from parallel cultures of yeast containing YEP 51 (or IP 51) are shown. Numbers that are vertically aligned in the same box are from experiments done on the same day.

galactosidase expression directed by a promoter containing UAS2UP1 is induced 5-6X by lactate compared to glucose (Forsburg and Guarante, 1988). In contrast, in two independently derived clones of yeast, containing RSVCAT integrated at the HIS3 locus in opposite orientations, the CAT activity per μg of total yeast protein in glucose was consistently greater than in lactate (Table 3).

Further, when FRSVCAT is used to transform BWG 1-7A and its derivatives hap2-1 (Guarante, 1984) which is HAP2$^-$, and BWG 1-7AΔhap3 (Olesen et al., 1987; Hahn et al., 1988) which is HAP3$^-$, the CAT activities in the 3 strains were 19.2, 12.8 and 16 respectively (% chloramphenicol acetylated/μg protein/min.). This too, is in marked contrast to the 50 fold less activity seen with UAS2UP1-beta-gal constructs in the HAP2$^-$ and HAP3$^-$ strains compared to BWG 1-7A (Forsburg and Guarante, 1988).

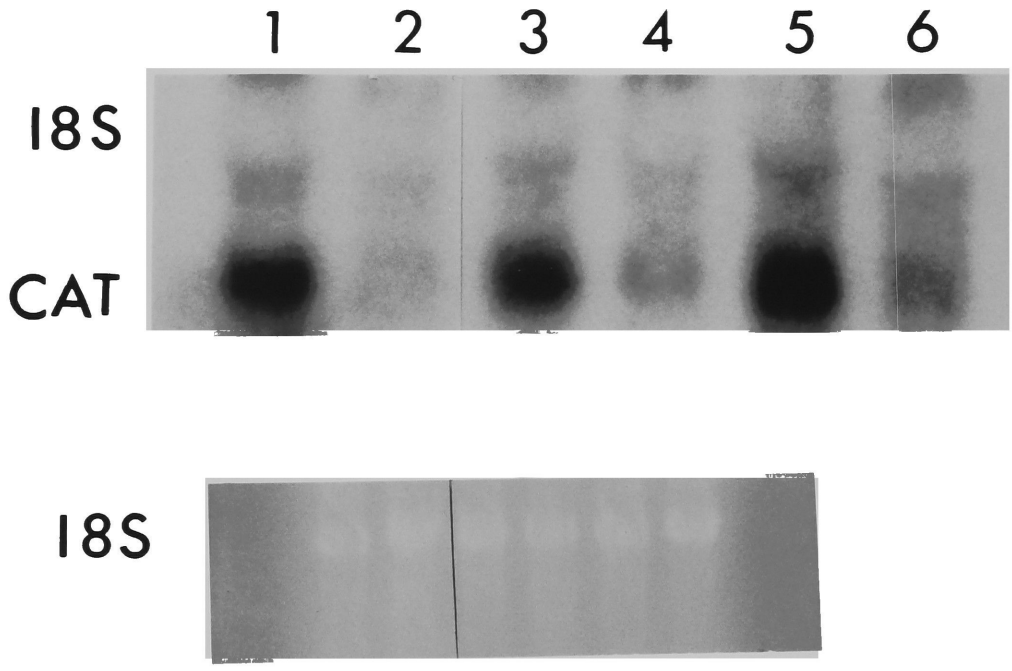
v-src may stimulate expression from the RSV LTR in yeast.

It has been shown that when v-*src* is expressed in yeast it is myristylated, goes to the plasma-membrane and has tyrosine kinase activity (Kornbluth et al., 1987; Brugge et al., 1987). Since, in animal cells, v-*src* appears to stimulate transcription from the RSV LTR using CCAAT boxes in the LTR, and since these same CCAAT boxes appear to play a role in transcription from the LTR in yeast, it was of interest to examine if v-*src* could induce expression from the RSV LTR in yeast.

Over-expression of v-*src* in yeast has been demonstrated to be toxic (Kornbluth et al., 1987; Brugge et al., 1987). Hence our strategy was to examine the effects of low levels of v-*src*. Towards this end, Y-vsrc or the control vector YEP51 was introduced into a strain of yeast harboring a single copy of RSVCAT at the HIS3 locus (RSVCAT-HIS). As expected, changing the medium to one containing galactose caused a dramatic loss of yeast proteins and CAT activity. However, even in media without galactose, the strain

FIGURE 19.

Northern blot analysis of total RNA from yeast containing one copy of RSVCAT integrated at the HIS3 locus (RSVCAT+HIS) and either IP 51 (lanes 1,2) or I-vsrc (lanes 3,4: clone K; lanes 5,6: clone L) at the LEU2 locus. $3x10^7$ yeast were inoculated into 20 ml of synthetic medium lacking histidine and leucine and with glucose as the sole carbon source. After 24 hrs., the cultures were split, spun down, washed, and resuspended in the same volumes of media containing either glucose (odd lanes) or galactose (even lanes). RNA was harvested after 16 hrs., blotted and probed with nick-translated RSVCAT. The major species of CAT RNA is shown. The lower panel shows the blot, stained with ethidium-bromide, to demonstrate that equal quantities of RNA were present in each lane.


1
2
3
4
5
6
18S
CAT
18S

containing Y-vsrc often showed a two-fold higher CAT activity than the one containing the control vector (upper half of Table 4). Brugge et al. (1987) have shown that there is leaky expression of v-*src* in yeast containing a plasmid analogous to Y-vsrc even in the presence of glucose. On one particular day, the level of activity was lower in Y-vsrc containing yeast compared to YEP 51 containing yeast, demonstrating that there is some variability in the system.

An alternative way to produce v-*src* in low doses would be to express it from a single copy integrated gene instead of a multicopy plasmid. The 2 μm ARS was excised from Y-vsrc and YEP 51 to give plasmids I-vsrc and IP 51. These were linearized at an unique *Cla* I site in the selectable LEU2 gene and transfected into yeast containing one copy of RSVCAT at the HIS3 locus (RSVCAT+HIS), which were then selected for LEU^+ phenotype. Several clones were grown under various conditions and CAT activites measured to check if v-*src* had any stimulatory effect on expression from the RSV LTR. The results presented in the lower half of Table 4 again suggest that under one particular condition a stimulatory effect of v-*src* on the LTR may be discerned. The requirement of growth in synthetic medium (as opposed to YEP medium) is obvious and could be due to a difference in phosphate content between the two media.

In an effort to ensure that the 2 fold increase in CAT activity in the I-vsrc containing yeast was at the RNA level, the experiment presented in Fig. 19 was done. Growth in galactose for 16 hrs. appears to suppress CAT RNA very strongly, but again it can be seen that both the clones of yeast carrying I-vsrc contain about twice as much CAT mRNA than the clone with vector alone. This difference is not seen when the same clones are grown in glucose.

DISCUSSION.

The RSV LTR is recognized by the transcription machinery of S. cerevisiae.

There have been a few reports in the past of promoters from animal cells directing transcription in yeast (Handa et al., 1985; Kornuc et al., 1988). However, this is the first instance of (a) a retroviral promoter directing transcription accurately in yeast and (b) the same TATA box and upstream activating sequences being used in animal cells and in yeast. The initiation of transcription about 60 bases downstream from the TATA box is expected from the studies of Handa et al. (1985) who observed a similar phenomenon when studying the initiation of transcription from the E1A promoter in S. cerevisiae. The S1 nuclease assay shown by Kornuc et al. (1988) is suggestive of a shift of 40 bases in the start-site of the adenovirus E3 promoter in yeast, compared to HELA cells (contrary to the conclusions of the authors).

How common is this phenomenon likely to be ? If initiation of transcription in S. cerevisiae requires only a TATA box, then it may be more likely than expected. The situation may be complicated somewhat if there are multiple TATA box like sequences, each recognizing a specific factor (Simon et al., 1988). It may be possible that some TATA box factors are absent in yeast. There may also be complications produced by sequences near the promoter fortuitously resembling binding sites of yeast DNA-binding-proteins which might then interfere with transcription-initiation. It could be concluded, however, that if one were interested in using genetic approaches to isolate transcriptional factors that interact with a promoter, it is certainly worthwhile to check the activity of the promoter in yeast.

Are there multiple CCAAT box binding factors in yeast ?

There appear to be multiple CCAAT box binding factors in animal cells (Dorn et al., 1987; Chodosh et al., 1988a; Hatamochi et al., 1988; Santoro et al., 1988; Paonessa et al., 1988; Gil et al., 1988) making it likely that the same is true for yeast. The CCAAT sequences in the RSV LTR behave as activating sequences in yeast, suggesting that they may bind CCAAT box factors. Possible candidates for such factors are the HAP2/HAP3 encoded proteins (Chodosh et al., 1988b). However, the fact that lactate does not induce the RSV LTR and that there is no significant decrease in LTR activity in hap2$^-$ or hap3$^-$ yeast, makes it unlikely that HAP2/HAP3 play a significant role in regulating transcription from the RSV LTR in S. cerevisiae. This, together with the observations in the following section, opens up the possibility that there are other CCAAT box binding factors in yeast.

Do the Ty elements of yeast use similar factors for directing transcription ?

S. cerevisiae contain retrovirus like elements known as Ty elements that belong to two classes Ty1 and Ty2. These have terminal delta sequences that are similar to the LTRs of retroviruses. Mutational analysis has shown that transcription from these delta elements require an intact TATA box, and an UAS located about 100 bases upstream from the TATA box (Liao et al., 1987). Inspection of the UAS region reveals the presence of a CCACT sequence.

It has also been shown that transcription from the delta elements is influenced by "internal enhancer sequences" situated in the "epsilon" region of the Ty element. One of these sequences from Ty1, which has been found to confer 2.5 fold stimulation of expression from heterologous promoters in haploid cells, and a fivefold repression in a/alpha diploid cells is shown in Fig. 18. This same element shows sequence similarity

to the a1-alpha2 site of MAT alpha and could be involved in the repression of MAT alpha in a/alpha diploid cells. This "Block II" region binds a protein present constitutively in haploid and diploid cells, which fails to bind if there is a mutation that also inactivates the "enhancer" like function of the sequence (Company and Errede, 1988). As shown in Fig. 18, the "Block II" region contains a CCAAT motif and Company and Errede (1988) have shown that mutations change either the CCAAT sequence or the purine that is present 5 bases downstream from the CCAAT element inactivate the ability of the element to both bind the DNA binding protein and to act as an "enhancer" in vivo. The significance of the purine in animal cells has already been discussed (Fig. 9).

Taken together, these observations underline the similarities between the Ty elements and the RSV LTR, and may explain why the LTR directs accurate initiation of transcription in yeast, and even why the same sequence motifs for UAS function are used that are used in mammalian cells.

Can this system be used to genetically define transcriptional factors that interact with the RSV LTR ?

The presence of phenotypic changes in yeast that correlate with changes in the strength of the RSV LTR (Fig. 17) makes the system amenable to genetic analysis. One can also use RSVNEO to confer resistance to G418 in yeast (data not shown) thus offering a second phenotype for screening for mutants with defects in the transcriptional machinery as opposed to mutants with lesions in the CAT gene or steps in the uptake, metabolism or action of chloramphenicol.

Further, a phenotype arising from absence of a TATA box or CCAAT box makes it very easy to (a) test for activity of various TATA sequences or CCAAT sequences and (b) test for the activities of factors binding to these sequences. For the CCAAT box

binding factors, one can hope to define new factors in yeast, either by screening yeast for mutants that have less activity from the RSV LTR, or by directly looking for yeast proteins (or genes coding for such proteins) that bind to the CCAAT sequences in the RSV LTR.

Does v-*src* stimulate expression from the RSV LTR in yeast ?

The results presented in this chapter can best answer this question with a "maybe". The twofold stimulation seen in some of the experiments may be significant, particularly because the CCAAT boxes appear to stimulate RSV LTR activity only about twofold in yeast (Fig. 16), as opposed to the tenfold stimulation by these same CCAAT boxes seen in mammalian cells (Fig. 3). Further, a twofold difference in CAT activity does give a difference in phenotype (Fig. 17) and should that be true in the case of the v-*src* stimulation of the RSV LTR, then the system would lend itself to genetic analysis.

The major weakness with this part of my thesis is that we have not been able to demonstrate an induction of CAT activity on switching the yeast containing RSVCAT and I-vsrc to a medium containing galactose (which would induce v-*src*). This may be due to a suppressive effect galactose itself has on transcription from the RSV LTR (Fig.21) in synthetic medium. In fact, within three hours of transferring yeast that carry RSVCAT and IP 51 into a galactose containing synthetic medium, a 50 % drop in CAT activity is seen. In YEP medium, in contrast, neither the suppressive effect of galactose nor the "inducing" action of v-*src* can be seen. Another way of interpreting this would have been that in synthetic medium, galactose represses the RSV LTR and v-*src* relieves this repression partially. However, this is made unlikely by the observation in Table 4 of a 2X stimulation of CAT activity by Y-vsrc in sugars other than galactose, namely, glu-

cose and raffinose. Anyhow, should there be a phenotype produced by the action of v-*src* on RSVCAT, it would be fruitful to look for mutations that interfere with the phenotype, on the presumption that such a mutant can indicate how v-*src* influences gene expression.

CHAPTER-5: EPILOGUE

In this chapter, I intend to discuss three issues not discussed earlier.

Is the effect of tyrosine kinases, including v-*src*, on a transcription factor likely to involve a tyrosine-kinase-specific pathway ?

Two different cis-acting elements from the rat prolactin and the Moloney murine leukemia virus promoters, which did not appear to bind a common factor, were both able to transfer the transcriptional effects of EGF and TPA (Elsholtz et al., 1986). Likewise, Fisch et al. (1989) reported that an AP1 binding site can mediate induction by EGF and TPA. Both these results indicate that the action of tyrosine kinases on transcription can (i) be mediated by different responsive elements and their binding factors, and (ii) the same responsive elements and factors can be stimulated by serine kinases like protein kinase C. This suggestion is reinforced by the observed convergence of several inducing pathways on the short "serum response element" (SRE) of the c-*fos* promoter. The SRE can mediate induction by agents like serum, EGF, and insulin on the one hand and agents like TPA on the other (Treisman et al., 1985; Gilman et al., 1986; Fisch et al., 1987; Greenberg et al., 1987; Stumpo et al., 1988). These observations imply that at least two tyrosine-kinases (EGF receptor and insulin-receptor) utilize final effectors that are common to TPA, which activates a serine kinase. Even among the serine-threonine kinases, two different pathways involving either the cyclic AMP dependent protein kinase or protein kinase C, appear to induce the same factor, namely AP2 (Imagawa et al., 1987). All of these results indicate that there is significant overlap in several of the growth promoting pathways, regardless of whether they start with a tyrosine kinase or a serine-threonine kinase.

A short sequence from the polyoma virus enhancer has been shown to confer inducibility by TPA, serum, and a variety of transforming oncogenes including polyoma middle T, v-*src* and v-*ras* to a heterologous promoter (Imler et al., 1988; Wasylyk et al., 1988). The sequence binds a mouse transcription factor, PEA1, though whether the inducing agents change the activity of the factor is not yet known. PEA1 is closely related to AP1. Both of them drive transcription from promoters of tumor viruses, and can confer responsiveness to a tyrosine kinase and to TPA (Angel et al., 1987; Lee et al., 1987; and references cited in preceding paragraph). The similarities between PEA1/AP1 and the CCAAT box binding factor we have described are inescapable, and suggest that the CCAAT factor which we find to be stimulated by tyrosine kinases, could also be induced by TPA or cAMP.

Finally, the stimulation of transcription of the AP1 gene by EGF (Quantin and Breathnach, 1988) further illustrates the interplay of the tyrosine kinase induced pathways and other signal transduction pathways.

Having considered all of the above, it does seem that the nuclear effectors of tyrosine kinases are unlikely to to be tyrosine-kinase specific. In fact, the convergence of tyrosine kinase pathways and those induced by protein kinase C might be more than a co-incidence. It may well be that one acts through the other. Considering the subcellular localisation of most of the tyrosine kinases in the plasma membrane, and of several serine kinases in the cytosol, e.g. the oncogene c-*raf* (Rapp et al., 1988), c-*mos* (Seth and Vande Woude, 1988) and even protein kinase C (Farrar et al., 1985; however, also see Nishizuka, 1988), it may be pertinent to suggest that the tyrosine kinases act by modulating the actions of the cytosolic serine kinases.

There is some experimental evidence to support this conjecture. Though the CCAAT box binding factor appears to be induced by tyrosine kinases, attempts to

deplete the factor from a nuclear extract using antiphosphotyrosine antibody were unsuccessful (our unpublished results, with M. Hamaguchi). Further, when nuclear extracts from 3Y1 cells and from HeLa cells were run out on a gel and immunoblotted with antiphosphotyrosine antibody, we found no enrichment of any of the phosphotyrosine containing proteins in the nuclear extract compared to the cytosolic fraction. This is consistent with the immunofluorescence data of Maher et al. (1985), who found most of the phosphotyrosine containing proteins outside the nucleus. The DNA binding activity of the "serum response factor" (SRF) which binds to the "serum response element" (SRE) upstream of the c-*fos* promoter has been shown to be induced by EGF in A431 cells (Prywes and Roeder, 1986) and yet, direct phosphoamino acid analysis of SRF labeled with radioactive orthophosphate from A431 cells revealed phosphorylation on serine and none on tyrosine (Prywes et al., 1988). All of the above would imply that direct phosphorylation of transcription factors by tyrosine kinases (Migliaccio et al., 1986; Auricchio et al., 1987) is more the exception than the rule. The phosphotyrosine containing nuclear proteins isolated from Abelson transformed fibroblasts (Bell et al., 1987) have been shown to bind to DNA and appear to have counterparts in RSV transformed CEF and 3Y1. These proteins may turn out to be transcription factors but there is no experimental evidence for this yet.

In contrast, there is plenty of evidence for the activity of transcription factors being regulated by direct phosphorylation of the factors themselves (Sorger et al., 1987; Hoeffler et al., 1988; Prywes et al., 1988; Sorger and Pelham, 1988), and in at least one instance, the phosphorylation is on serine and not tyrosine (Prywes et al., 1988). Likewise, there is ample evidence for transcriptional factors being activated by serine-threonine kinases in the cell (Comb et al., 1986; Sen and Baltimore, 1986; Imagawa et al., 1987; Angel et al., 1987; Lee et al., 1987; Riabowol et al., 1988; Fisch et al., 1988;

Hyman et al., 1989; Fisch et al., 1989;). Finally, at least some serine-threonine kinases are known whose activity is stimulated by tyrosine kinases (Morrison et al., 1988; Sturgill et al., 1988). All of which might be construed to suggest that tyrosine kinases act through serine-threonine kinases to modulate the activity of transcription factors.

There is some pharmacological evidence to support this point of view. Hall and Stiles (1987) showed that depletion of protein kinase C from a cell by prolonged exposure to TPA, blocked the induction of c-*myc* and c-*fos* by PDGF.

This line of reasoning is also supported by the fact that while v-*ras* transformed cells and v-*mos* transformed cells are as serum-independent as cells transformed by tyrosine-kinases like v-*src*, there is no increase in the levels of PTYR containing proteins in the former (Mayer et al., 1988b).

Transport of proteins into the nucleus.

The incidental finding that serum causes a tenfold increase in extractable nuclear proteins even in the presence of cycloheximide, points to protein import into the nucleus (reviewed in Gerace and Burke, 1988) as an important step in the control of activity of transcription factors. A basic stretch of amino acids has been identified as a nuclear localization signal in several nuclear proteins. Furthermore, systems that carry out transport of nuclear proteins into the nucleus in vitro have been developed, and studies with these have revealed that uptake by the nucleus involves a binding step which requires the presence of the karyophilic signal mentioned above, followed by an ATP dependent transport step (Newmeyer and Forbes, 1988). Hence, transport of transcriptional factors into the nucleus could be a regulatable process.

Another possibility could be that transport into the nucleus is regulated by factors in the cytosol which form complexes with the transcriptional factors and prevent their

import into the nucleus. Such a mechanism may underly the post-translational activation of NFkB by TPA (Bauerle and Baltimore, 1988a and 1988b). It would be fascinating if some of the repressors identified genetically in S. cerevisiae, which appear to act by binding to positively acting transcription factors, e.g. GAL 80 binding to GAL4 or PHO 80 and PHO 85 binding to PHO 4 (Oshima, 1982), are similar to IkB, which acts by binding and inactivating NFkB in the cytosol of animal cells (Bauerle and Baltimore, 1988a, 1988b) because that would indicate that this mechanism of control is very widespread, and open up another way to regulate the activity of transcription factors.

Signal transduction in Saccharomyces cerevisiae.

The homology of the transcriptional apparatus of yeast and animal cells has been highlighted in the recent past (Chodosh et al., 1988b, 1989; Fodor et al., 1988; Cavallini et al., 1988; Buratowski et al., 1988; Metzger et al., 1988; Webster et al., 1988;). A natural question that arises from these observations is how homologous are the signal transduction systems ? Celenza and Carlson (1986) reported that derepression of a glucose repressible gene, SUC2, requires the activity of SNF1, which codes for a serine threonine kinase. SNF1 may be acting through a negative regulator, SSN6. Another signal transduction system that has been studied in great detail has been the response to mating factor. Here too, some genes that are essential for normal transduction of the signal code for proteins with counterparts in animal cells. The receptors for the mating factors, STE2 and STE3, resemble beta adrenergic receptors with seven hydrophobic domains (Nakayama et al., 1985; Hagen et al., 1986). The pathway also utilizes a gene coding for an alpha subunit of a G protein called SCG1 or GPA1 (Dietzel and Kurjan, 1987; Nakafuku et al., 1987), and genes coding for beta and gamma subunits for G proteins called STE4 and STE18 (Whiteway et al., 1989).

In some instances where genes for DNA binding proteins have been cloned from animal cells and introduced into yeast, they have retained their function. Of particular interest is the ability of the human oestradiol receptor to activate gene expression in a oestrogen dependent manner through an oestrogen responsive element in yeast (Metzger et al., 1988; Schena and Yamamoto, 1988) and the ability of the bovine papilloma viral E2 trans-activator to activate transcription from E2 binding sites also in yeast (Lambert et al., 1989). All of these findings made us examine more closely if the transcriptional effects of the v-*src* tyrosine kinase could be analyzed in yeast. The results of these experiment are discussed in chapter 4. Here I wish to address the phenotype of one particular clone of S. cerevisiae (called GOURI) expressing v-*src* under control of a GAL10 promoter (which is repressed by glucose, derepressed by raffinose, and induced by galactose).

GOURI had a growth-rate in YP-glucose and YP-raffinose that was approximately the same as the clones containing vector alone (doubling time : 2 hrs.) but the saturation density was 1/2 as much. However, in YP-raffinose and in YP-galactose, GOURI takes on an elongated morphology reminiscent of "schmoos". It was possible that the growth that was seen with GOURI in YP-raffinose was due to cells that shut down v-*src* expression. The experiments were repeated in synthetic medium with selection for the LEU^+ phenotype (the v-*src* plasmid carries a LEU2 gene). Here the schmooing phenotype of GOURI was more pronounced in galactose than in raffinose and was again not seen in glucose. Growth in synthetic medium with raffinose was slow for GOURI (doubling time : 4 hrs.), compared to that for clones with vector alone (doubling time : 2.3 hrs.) or another clone expressing v-*src* (doubling time : 2.0 hrs.). The saturation density of GOURI in synthetic medium with galactose was tenfold less than that of yeast with vector alone and half of that of other clones containing the integrated v-*src* plasmid.

What does all this mean ? Since, the phenotype of GOURI was not found in five other clones containing I-vsrc, I conclude that GOURI has another mutation which causes v-*src* to confer the "schmooing" phenotype. It is likely that v-*src* has a role to play in the phenotype, because of its induction by raffinose and galactose, but this has to be proved genetically. Should this be the case, then GOURI may provide a novel approach for studying the action of v-*src* in yeast. Particularly in light of G protein involvement in the signal transduction of the pheromone response pathway (which does produce schmoos), it is possible that the involvement of v-*src* in inducing the formation of schmoos, reflects the natural role of a yeast tyrosine kinase in this pathway.

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