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V-*src*-induced-transcription of the avian clusterin gene

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ABSTRACT

We have isolated the avian gene T64 corresponding to the mammalian clusterin, on the basis of high accumulation of its template mRNA in cells infected with oncogenic retroviruses. Since the clusterin was shown to have a protective effect against the immune system, its induction by oncogenic viruses is of major biological importance. The unique, short 5 kb-long T64 genomic locus is inactive in normal quail embryo fibroblasts in primary culture whereas it shows a high transcriptional activity after transformation by the Rous sarcoma virus. The 963 bp-long 5′ flanking region is sufficient to drive the transcription of the chloramphenicol acetyltransferase reporter gene in a thermodependent manner when a thermosensitive version of pp60$^{v\text{-}src}$ is used. Deletion and point mutation analyses of the promoter show that the v-*src* response requires at least two separate elements : PUR and AP-1, located respectively at positions −167 to −152 and −25 to −19 relative to the single transcription initiation site. In addition, the binding of specific nuclear factors to these responsive elements correlates with the T64 promoter activation.

INTRODUCTION

Infection of avian cultured cells with the Rous sarcoma virus (RSV) causes deep genetic changes of the host cells. Somes cellular genes are repressed (1), while expression of others is stimulated by RSV. We have found that the gene most strongly overexpressed upon RSV infection in neuroretina cells and named T64 (2) is analogous to a mammalian gene, isolated in various and independent contexts (for reviews, see 3; 4), and whose gene product displays multiple activities : it is capable of binding plasma membanes (5; 6) (clusterin), secretion vesicles (3; 7; 8) (secretogranin IV) and circulating lipids (9) (Apolipoprotein J); it can interact with immunoglobulins (10) and shows structural (11) and functional (11; 12) relationships with cytolytic molecules of the membrane attack complexes. In the immunological context, clusterin has been termed cytolytic inhibitor (12)(CLI).

Interestingly, overexpression of this gene has often been found associated with the triggering of programmed cell death (13,14,15) and with severe physiological disturbances including the kidney or brain degenerative pathologies, such as glomerulonephritis (11), kidney allograft rejection (16) and Heymann nephritis (17) or scrapie (18) and Alzheimer's disease (18,19). In this last case, clusterin was shown to be a constituent of the β-amyloid plaques (20), and is specifically accumulated into dystrophic neurones (21). Some of these observations sugest a predominant protective role against cytolysis (22; 23; 24; 25). In this respect, the dramatic induction of this gene by various oncogenes (2), may provide a clue to the tumorigenic fate of oncogene-transformed cells.

In order to get insight into the mechanisms of induction of this gene by oncogenes, we have isolated the genomic locus corresponding to the T64 gene and analysed the transcriptional activity of its 5′-flanking region. We show that the 963 bp-long proximal sequence is a TATA-less promoter, which directs T64 mRNA transcription from a single initiation site. The T64 transcriptional induction caused by RSV infection is strictly dependent on the presence of an active *src* oncoprotein and requires both an AP-1 consensus site and a purin rich element named PUR. Because of the fundamental role of the AP-1 site in mediating effects of the cell proliferation control genes (*jun/fos*), and of the association of Pur elements with DNA replication, our work proposes a link between mitogenic signals and T64 transcription. In view of its use as a cell death marker, these findings are in support to the model suggesting that cell suicide processes through an abortive mitosis (26), and that cell death and proliferation share common signal mechanisms (27), as indicated by the prostate programmed cell death model which has led to the isolation of clusterin (TRPM-2) (14, 27). This work presents evidence, at the molecular level, for the emerging idea that apoptosis and oncogenesis are two facets of a balanced genetic program (28, 29).

MATERIALS AND METHODS

Cell cultures and viruses

Quail embryo fibroblasts (QEF) were prepared from 7 day-old japanese quail embryos (*Coturnix coturnix* Japonica). They were maintained and infected as previously described (2). The wild type and mutant strains of RSV were respectively SRA and *ts*NY68 (2), whereas the transformation defective virus was the RSV progeny of the derived RCAS retroviral vector devoid of the v-*src* oncogene (30). QEF were transfected using the polybrene-dimethylsulfoxide transfection technique (30).

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Cloning and sequencing of the T64 5'-flanking sequence

The T64 genomic locus was isolated from a λEMBL3 quail genomic DNA library, probed with a nick-translated, ^{32}P-labeled, T64 full-length cDNA (2). A NcoI-NcoI fragment, corresponding to the 5' region of the T64 gene, was isolated and first subcloned into the NcoI site of pGEM-5ZF (Promega). A 1 kb-long SstI-SstI fragment from this construction, including the SmaI-NcoI fragment of the T64 promoter and corresponding to nucleotides −963 to +55 relative to the transcription start site (Figure1), was subcloned into the SstI site of pBSK$^+$ (Stratagene). This construction, called pT64 was subjected to two series of unidirectional deletions, carried out using the α-thio-nucleotide protection and ExoIII-S1 nuclease technique. Inserts shortened in each orientation were sequenced by the dideoxy chain termination technique adapted to supercoiled plasmids with (α^{35}S) dATP (Amersham) and T7 DNA polymerase (Pharmacia). They are numbered relative to the most distal 5' nucleotide of the deleted promoter.

Determination of the transcription start site by primer extension

Two 20-mer oligonucleotides complementary to the regions +22 to +41 and +94 to +113, located respectively upstream and downstream from the translation initiation site, were end-labeled with T4 polynucleotide kinase and (γ^{32}P)ATP (Amersham). 3μg aliquots of poly A$^+$ RNA from RSV-transformed cells were heated at 65°C for 5 min, chilled on ice and annealed to each labeled oligonucleotide at 37°C for 5 min in a 2× reverse transcriptase mixture (0.1M Tris−HCl pH:8.5, 20mM $MgCl_2$, 0.1M KCl and 2mM dithiothreitol). DNA synthesis was carried out with the four dNTPs, each at a final concentration of 300μM, and the above oligonucleotides served as primers for DNA polymerisation with 15 units of avian myeloblastosis virus reverse transcriptase (Boehringer) at 42°C for 45 min in a final volume of 30 μl. Dideoxy DNA sequencing reactions of a plasmid containing the T64 gene were done using the same unlabeled primers, (α^{35}S) dATP, and they were run in parallel with 3μl aliquots of the above primer extensions in a 6% polyacrylamide sequencing gel.

Assays for transient expression of T64 promoter-CAT constructs

HindIII-AccI fragments from the original and the deleted pT64 plasmid, containing either the SmaI-NcoI fragment or ExoIII-5' shortened versions of the T64 promoter, were subcloned in the HindIII and AccI sites upstream from the CAT-coding sequence present in a pBLCAT2 plasmid (kindly provided by B. Luckow) previously deleted of its HSV-tk promoter by a BglII+BamHI digestion and religation. The 1 kb-long and ExoIII-5' shortened fragments of the promoter were then tested in CAT assays. For the pΔ(−175, −145)CAT2 internal deletion construct, a −963, −176 SmaI-EcoRV fragment was ligated in the HindIII-blunted site of p(−144, +55)CAT2. RSV-infected or non infected QEF (1.5×10^6 cells per 100 mm petri dish) were transfected with 5μg of T64 promoter-CAT plasmid and 4μg of the pIRV β-galactosidase expression plasmid (31). After 24 hours, cell cultures were divided in two lots and were either maintained at 37°C or transferred at 41°C for 36 hours to inactivate the *ts* v-*src* oncoprotein (32). Cell lysates were prepared as described (32) and they were normalized for transfection efficiency using the β-galactosidase activity (33) before CAT assays. In our conditions, transfection efficiency varied only within narrow limits (less than 1.5 fold range). The plates were autoradiographied, and radioactivity was determined by liquid scintillation counting. The CAT activities were quantified by the ratio:acetylated/total chloramphenicol. The background was determined with the pBLCAT2 plasmid deleted from its thymidine kinase promoter. All test plasmids were analyzed in triplicate through at least three separate experiments. In these conditions, the results varied by 20% or less.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from 10^8 exponentially growing cells and gel retardation experiments were carried out as described (32). The probes were end-labeled with T4 polynucleotide kinase and (γ^{32}P) ATP (Amersham). For each assay, crude nuclear extracts (2.5μg of protein,) were incubated for 10 min at 4°C in 10mM HEPES pH:8.0, 50mM KCl, 1.5mM $MgCl_2$, 0.1mM dithiothreitol, 15% glycerol, 50ng of poly (dI-dC) and 2.5μg bovine serum albumine. Then, 2×10^4 cpm (approximatively 0.25ng) of radiolabeled DNA fragments were added, binding reactions were allowed to proceed for 15 min at the indicated temperature, and they were electrophoresed at 4°C, 150V, for 1.5 hours through a 15 to 30 cm long 4.5% nondenaturing gel (80:1 acrylamide:bisacrylamide) containing 0.2× Tris−boric acid−EDTA. Gels were dried and exposed overnight using hyperfilm MP (Amersham) and an intensifying screen. If needed, an excess of unlabeled competitor were added prior to the addition of the probe. The single stranded oligonucleotide used as a non specific competitor in the experiment presented on figure 5 corresponds to a region of the T64 gene coding strand, between nucleotides +126 and +146.

Production of *jun-fos* heterodimers *in vitro*

Proteins were synthesized *in vitro* using pGEM-7 vectors containing the full-length cDNA of murine c-*jun* and c-*fos* (kindly provided by M.Castellazzi and J.Ghysdaël) linearized at their 3' end. Capped RNAs were synthetized using standard conditions as recommended by the supplier (Promega). *In vitro* transcribed c-*jun* ands c-*fos* RNA were used to initiate protein synthesis in a messenger-dependent rabbit reticulocyte lysate (Promega) with ^{14}C-Leucine. The *Jun-Fos* heterodimerisation of *in vitro* synthesized products occurs during cotranslation (A.Sergeant, personal communication).

In vitro mutagenesis of the AP-1 consensus binding site

The AP-1 site located between positions −25 and −19 was mutated by using the site-directed mutagenesis kit from Biorad with a single-stranded oligonucleotide 5'-GGGC-ACGGGGCCGTCTAGAGGCAGCGGGCACCG-3' phosphorylated with T4 polynucleotide kinase prior to annealing. The expected site mutation was confirmed by DNA sequencing. The T64 promoter carrying a mutated AP-1 site, named AP-1$^-$, was then subcloned upstream of the CAT coding sequence.

RESULTS

Molecular cloning and sequence determination of the T64 5'-flanking region

We have isolated the T64 genomic locus by screening a λ EMBL-3 quail genomic library with the α^{32}P-labeled T64 cDNA probe (2). The positive phages were mapped by southern blot analysis. One which hybridizes with the 5' and the 3' probes derived from the full-length cDNA was assumed to contain the

complete T64 transcribed sequence and a 2 kb long 5′ flanking region. This latter fragment has been subcloned into the pGEM-5 sequencing plasmid. A 1 kb-long SmaI-NcoI fragment containing the 55 bp-long leader sequence adjacent to the NcoI site, was subcloned in pBSK and sequenced. Nucleotide numbering is referred to the +1 transcription initiation site determined below.

The sequence shows that the 5′-flanking region of the T64 gene lacks a classical TATA element and is 59% G+C rich between positions −700 and −1. It contains a typical CCAAT box located at position −83 to −87 in the reverse orientation (figure 1). Since the 1kb-long DNA sequence is sufficient to confer optimal expression of a reporter gene (see below), we have searched for potential binding motifs for transactivator proteins. Three regions match the AP-2 consensus GCCNNNGGC/G (34) at positions −625 to −617, −339 to −331 and −54 to −46. Such AP2 binding sites mediate transcriptional activation by both protein kinase C and protein kinase A (35). The region −89 to −80 is identical to the TGF-β inhibitory element (TIE), which can bind a c-*fos* containing complex (36). The proximal sequence −25 TGACTCA −19 is the canonical recognition site for the transcription factor AP-1 (37).

Additional experiments (see below) have led us to focus attention on the −167 to −152 domain that we have named PUR because it exactly matches, on its complementary strand, a so-named holopurin domain identified previously in the promoter of the human c-*myc* gene and associated with an origin of replication (38).

Another prominent feature of the T64 promoter is the highly monotonous sequence (TTCCC) repeated 48 times and located from nucleotide −932 to −693 upstream of the start site (figure 1). This repetitive sequence is totally asymmetrical in base distribution, exclusively composed of purines in one strand and of pyrimidines in the other. We have recently demonstrated that this poly(pyrimidine)-poly(purine) sequence with repeated motifs may adopt pH- and divalent ion-dependent triple-helix structures *in vivo* (39).

Fine mapping of the transcription start site

Absence of a TATA box often correlates with the co-existence of several scattered transcription start sites in the promoters of constitutively expressed genes (40). We have checked for such a possibility and have determined, by primer extension analysis, the initiation sites of T64 mRNA transcription. The primer extension method was preferred to the S1 protection analysis because avoiding the heterogeneity of fragment lengths due to an activity in S1 preparations that attacks RNA-DNA hybrid termini (41). Two different oligonucleotide primers were hybridized to poly A+ RNA purified from quail neuroretina cells transformed by RSV (2). They were each elongated in the

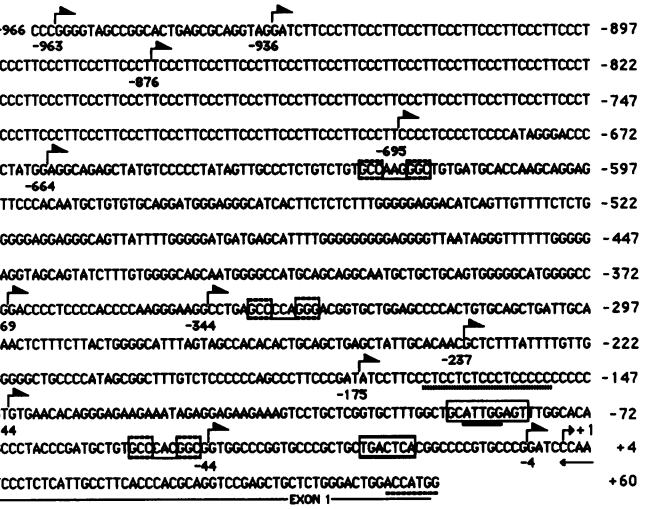

Figure 1. T64 promoter sequence. The DNA sequence of the most proximal 963 bp of the quail T64 promoter is described. Numbering is referred to the +1 transcription initiation site. The different potential binding sites for transcription factors are indicated: CCAAT box (underlined), TGF β inhibitory element (plain box), AP-2 (dashed box), and AP-1 (bold box). The PUR domain is underlined with small dashed line, the displayed strand is complementary to the consensus sequence identified in the human c-*myc* promoter (38). The Kozak translation initiation consensus sequence is underlined by a large dashed line. The position of the first nucleotide of each deletion is indicated by an arrow.

Figure 2. Mapping of the transcription initiation site by primer extension. As indicated in Material and methods, polyA$^+$ RNA from RSV-infected cells were hybridized to (α ^{32}P) labeled oligonucleotides annealed to positions +22, +41 (oligo 1) or +94, +113 (oligo 2) of the T64 mRNA. For accurate determination of the +1 start site, the same oligonucleotides were also used for priming dideoxy sequence reactions of a genomic DNA fragment encompassing the 5′ end of the T64 gene. The products of reverse transcriptase extension and the sequencing reactions were run on parallel (lane p) on the same 6% polyacrylamide, 7 M urea gel and they were analysed by autoradiography after overnight exposure.

presence of reverse transcriptase. A unique cDNA fragment is observed in both cases, which implies that T64 mRNA transcription is initiated at a unique site (figure 2). The two oligo-primed cDNAs migrate at a unique position coincident with the last C residue of a BamHI restriction site GGATCC.

Transactivation of the T64 upstream region by pp60$^{v\text{-}src}$

We have studied the ability of the T64 promoter sequence to drive expression of the CAT gene in a transient expression assay. For this purpose, the genomic fragment SmaI-NcoI corresponding to the 1 kb-long genomic sequence adjacent to the first exon and containing the 55 bp-long leader, was inserted in the CAT plasmid pBLCAT2 lacking distal and proximal promoter elements. Figure 3 shows that the construct does not lead to significant CAT activity in QEF grown either at 37°C or at 41°C. In contrast, when this construct is transfected in the same cells infected with the wild type RSV-A, a 200 fold increase of CAT activity is observed. This effect is not a mere consequence of retroviral infection since in the same conditions, the RSV progeny derived from the RCAS-A retroviral vector (30) containing no v-*src* oncogene does not drive expression of the CAT gene from the T64 promoter.

To further investigate the function of the active v-*src* oncoprotein on T64 gene expression, we have tested the effects of a thermosensitive form of this oncogene. QEF transformed with the thermosensitive v-*src* mutant strain tsNY68 of RSV were transfected with the T64-CAT construct. CAT activity was determined in extracts of cells grown either at the non-permissive (41°C), or at permissive temperature (37°C) for pp60$^{v\text{-}src}$ activity. The CAT activity becomes strikingly thermodependent in *ts*RSV-infected cells, decreasing by a factor of 55 at 41°C, but remains more or less constant at both temperatures in wt RSV-infected cells (figure 3). This indicates that T64 promoter activity

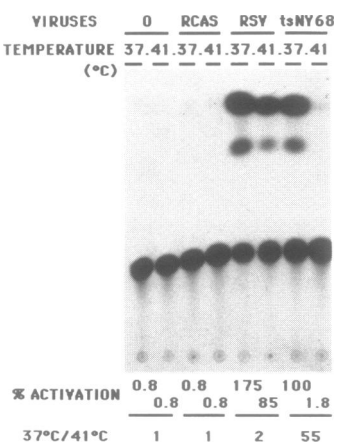

Figure 3. Transcriptional activation of the T64 promoter by pp60$^{v\text{-}src}$. The quail genomic DNA fragment SmaI-NcoI, located between positions −963 to +55 relative to the transcription start site was fused to the CAT gene in the plasmid pBL-CAT-2. 5μg of this construct were transfected in RSV-infected QEF. CAT assays were carried out with protein extracts from QEF cultured for 36 hours either at 37°C or 41°C as indicated, and infected or not with the following viruses: RCAS autoreplicative retrovirus derived from RSV SR-A, but deleted of v-*src*; wild-type SR-A strain of RSV; *ts*NY68 transformation mutant strain of RSV. For calibration of the transfection efficiency, all cell cultures were simultaneously transfected with 4μg of pIRV, a plasmid containing the β-galactosidase gene under the control of the constitutive β-actin promoter (4). CAT activation measured with tsNY68 RSV infected QEF at 37°C was arbitrarily set as 100%.

is strictly correlated with the transforming activity of pp60$^{v\text{-}src}$. We have then undertaken the functional dissection of the T64 promoter in order to delineate domains mediating the v-*src* response.

5′ to 3′ progressive deletion analysis

A series of progressive deletions, from nucleotides −963 to −1 was tested to isolate these cis-acting elements. As shown in figure 4, the removal of the long holopyrimidic tract (TTCCC)n (−963 to −664) has no detectable influence on the transcription efficiency. Longer deletions progressing untill nucleotide −236 has no determinant influence on CAT expression. But when the promoter is reduced to the 144 most proximal nucleotides, the transcriptional activity is strongly decreased. To localize more precisely the cis-acting element whose removal impedes the promoter activity, an intermediate deletion was carried out and showed that the active region spans from nucleotides −175 to −144. Its core sequence on the complementary strand is 5′-GGGGGAGGGAGAGGAG-3′, and exactly matches the PUR consensus 5′-GGNNGAGGGAGARRRR-3′ identified previously (38). The removal of nucleotides −144 to −44 leads to an additional loss of promoter activity (figure 4). The CCAAT box and the AP-2 element present in this region are likely to explain this observation.

Internal deletion of the PUR element

To further ensure the influence of the PUR sequence on T64 gene transcription, the −175 to −145 region encompassing the PUR consensus domain was removed, other promoter sequences remaining unchanged. This limited internal deletion leads to a 50% decrease of the promoter activity, as monitored by CAT-assay (Figure 3 bottom), confirming the role of the PUR element revealed by the progressive deletions. To investigate the role of more proximal sequences including the AP-1 consensus binding site, the PUR domain was kept intact and we have proceeded by point mutation analysis (see below).

Binding of single strand-specific nuclear factors to the PUR element correlates with T64 promoter activity

Using gel shift assays, we have first studied the ability of nuclear extracts from RSV-infected QEF to bind to the synthetic double stranded PUR domain, but without obtaining any binding (figure 5A). This result appeared surprising in view of the major functional influence of this cis-acting element. Following the approach used for studying a similar domain (38), we have tested the binding activity of the same nuclear extracts to each of the separated strands of the (−173, −151) PUR containing region. Figure 5A shows that purin and the pyrimidin strands form distinct complexes with nuclear factors. The complex observed with the purin probe can be self-competed (figure 5B), while those obtained with the pyrimidin probe cannot. In addition, the pyrimidin complexes are not longer observed if the electrophoresis is performed at 20°C (not shown), indicating that these complexes are neither specific nor stable. Since the double stranded probe fails to form any complex, the removal of the PUR binding by the complementary pyrimidic strand (figure 1B) can be interpreted more as a titration of the PUR probe rather than as a classical competition phenomenon. Finally, we have reliably observed that the PUR complex is 3 fold more abundant with nuclear extracts from *ts*NY68RSV-infected cells grown at 37°C which express T64, than with those from cells grown at

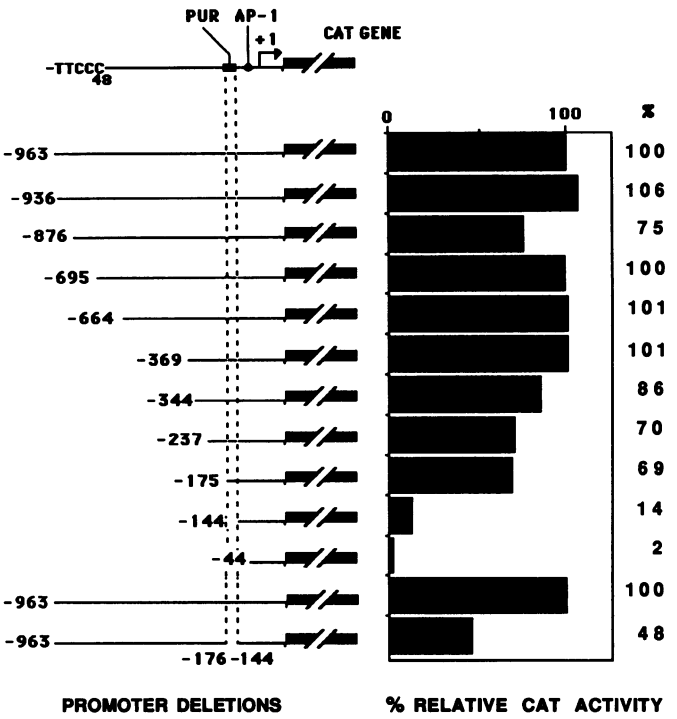

Figure 4. Deletion analyses of the T64 promoter. The relative transcriptional activities of 5′ deletion mutants tested in CAT assays are reported in the column on the right. All mutants retain the +1 transcription start site and the whole leader sequence. 5μg of these different constructs were transfected in RSV tsNY68 infected QEF grown at 37°C, with 4μg of pIRV as an internal control. The 100% activation was set for the 963 bp-long promoter, and the background was determined with a promoter-less CAT plasmid. These values were obtained by scintillation counting of chloramphenicol spots, and represent the average of six experiments. The bottom lane represents an internal deletion targetted to the PUR domain.

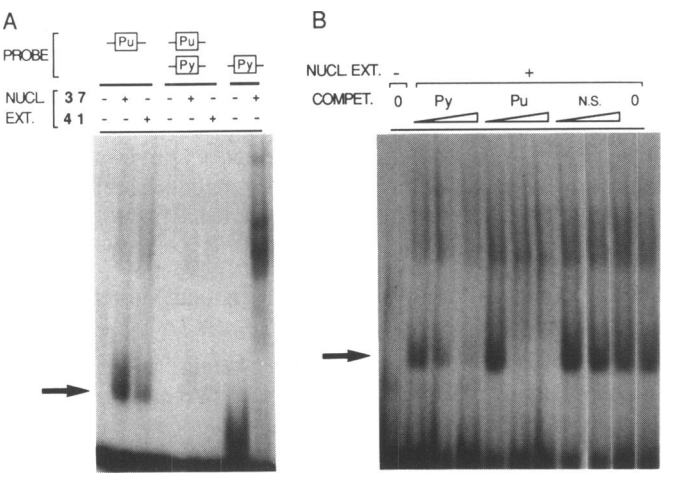

Figure 5. Gel mobility shift assays of nuclear protein binding to the PUR domain. **(A)** Comparative binding of nuclear extracts from tsNY68 RSV-infected QEF grown at 37° or 41°C, to the purin strand, the pyrimidin strand and the double strand, corresponding to the PUR domain. **(B)** Competition for single strand PUR binding. Indicated by enlarging open boxes, increasing concentrations of 10, 50 and 100 fold excess relative to the probe molarity were tested for each competitor. Py, Pu and N.S. refer respectively to the pyrimidine, the purine, and non specific single stranded oligonucleotides. Position of the PUR complexes are indicated with arrows.

41°C, where the T64 gene is silent. This close correlation supports an active function of the PUR factor in the T64 promoter activation.

The TGACTCA motif is necessary for the *v-src* response

To analyse the possible requirement of the AP-1 binding site on the v-*src*-induced T64 transcriptional activation, we have constructed a CAT reporter plasmid containing the complete T64 promoter sequence (−963, +55) with a mutated AP-1 site. In this plasmid, the −25 TGACTCA −19 motif is replaced by the TCTAGAC sequence, with the same global base composition. When tested in the same conditions as the wild type sequence, this mutation causes a 77% decrease of the CAT activity (figure 6A). Thus the AP-1 site is required for optimal v-*src* induction, by allowing the effects of upstream elements.

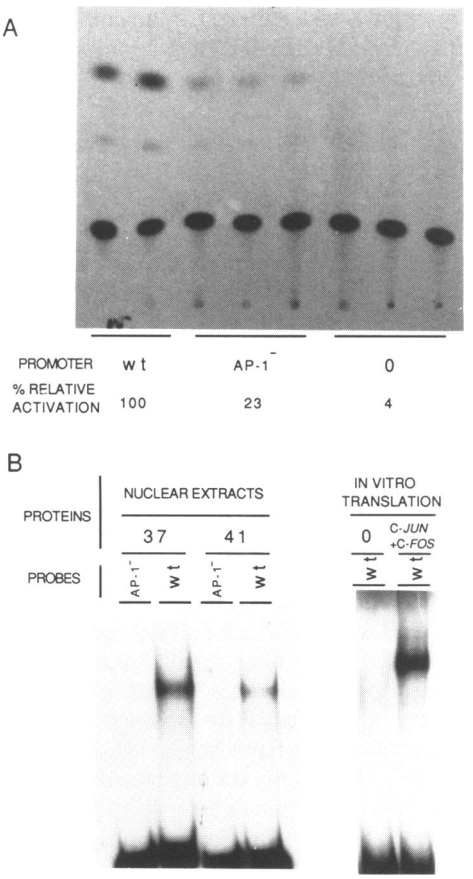

Figure 6. Role of the AP-1 binding site. **(A)** Comparative analysis of the CAT reporter gene expression driven by the wild type (wt), and the AP-1 directed mutant (AP-1⁻) of the T64 promoter sequence. 5μg of the constructs were transfected with pIRV, as an internal control in 1.5×10^6 QEF. 36h later, cells were harvested and β-galactosidase activity was determined prior to CAT assay for calibration. Estimation of the CAT activity was performed by liquid scintillation counting. Results are presented as duplicate or triplicate of the CAT assays. The CAT activity synthesized from the wt promoter was set as 100%. **(B)** Binding of nuclear extracts and of *in vitro* synthesized *Jun/Fos* proteins to the T64 AP-1 sequence. The T64 promoter fragment spanning from nucleotide −44 to +55, was mutated (AP-1⁻) or not (wt) in its −25 to −19 putative AP-1 binding site. EMSA were carried out after incubation with nuclear factors from v-*src*-transformed (named 37) or non-transformed (named 41) QEF, or with c-*Jun*/c-*Fos* proteins cotranslated *in vitro*. As a control, RNA-free lysate was incubated with the radioactive wt probe (lane o). EMSA were performed as described in Materials and Methods.

Binding of the AP-1 complex to the T64 promoter

We have also compared the relative ability of a minimal promoter region (−44 to +55) containing the wild type and mutated AP-1 sites, to complex with nuclear extracts from RSV-transformed QEF. The gel retardation assay presented on figure 6B shows that specific proteins bind to the wild type probe but not to the mutant TGACTCA motif. This suggests that the retarded complex results from AP-1 proteins/DNA interactions.

In addition, it must be noted that, as shown in figure 6B, nuclear factors binding the (−44, +55) region are more abundant in extracts from fully transformed cells (37°C) than in those from cells containing an inactive form of v-*src* (41°C)

Since the c-*jun* and c-*fos* family of oncoproteins are components of the AP-1 complex (42), it was of interest to check if these oncoproteins could account for the complexes observed with the AP-1 site in the T64 promoter sequence. As shown on figure 6B, c-*jun*/c-*fos* proteins cotranslated *in vitro* do form a retarded complex with the (−44 to +55) fragment. As a control, the control reticulocyte lysate containing no mRNA templates does not form any complex (figure 6B).

DISCUSSION

Clusterin is considered so far as the best genetic marker for cell death in higher vertebrates (26); the clusterin gene has been proposed as a model for understanding apoptotic regulation of gene expression (25). This first presentation of genomic regulatory regions of the clusterin gene already shows interesting features.

Control of transcriptional initiation in absence of a TATA box

The major feature of the T64-clusterin gene is the extremely low level of its expression in normal QEF cells as well as in most cells *in vivo*, as indicated by *in situ* hybridization experiments (data not shown). In this respect, we were first surprised to note that the promoter sequence is devoid of TATA box. Indeed, absence of a TATA box has been initially considered as restricted to genes expressed at low but constant level in all cells and with multiple transcription start sites. Recently however, a lot of evidences have accumulated for absence of this element in promoters of precisely regulated genes such as tissue-specific and inducible genes. The proposed functions of the TATA element are the precise determination of an appropriate start site and the synergy of interaction with upstream regulatory elements. Now, several elements are known to functionally replace the TATA element : initiators (40), non canonical TATA elements (43) and enhancer elements when localized in the vicinity of the initiation site (44). The yeast factor GCN4 with binding properties similar to AP-1 is known to activate transcription in a TATA-independent manner and is capable of interacting directly with RNA polymerase II in vitro (45). The very proximal localization of the AP-1 binding site in the T64 promoter is likely to reflect such a 'TFIID function' of AP-1 during T64 transcription.

The stringent transcriptional control of this gene can explain why the differential screening technique, based on the differential expression of genes between two physiological states, has led to four independent isolations of the clusterin gene, from quail (2), rat (13), hamster (18) and human (19). The T64 gene is thus a novel carefully regulated, but TATA-less gene.

AP-1 is required but not sufficient for full T64 transactivation by *v-src*

The binding of specific complexes like AP-1 on regulatory sequences can ensure a determinant role on the initiation of transcription, by itself or in cooperation with other elements. The stimulation by v-*src* of the T64 transcription clearly belongs to the second situation. On the one hand, the proximal promoter −144, +1 containing the AP-1 site is unable by itself to mediate the full v-*src* transactivation as demonstrated by 5′ to 3′ progressive deletions. But on the other hand, directed mutation of the single domain TGACTCA in a large promoter causes a 77% decrease of the T64 induction by v-*src*. AP-1 binding site could relay upstream v-*src*-dependent transactivation elements. Besides, the *Jun-Fos*/AP-1 complex is already known to play an important role in mediating gene expression in RSV-infected cells (42). The presence of AP-1 binding sites appears as a common feature shared by promoters of v-*src* inducible genes such as TGF-β (46), transin/stromelysin (47) or cytokine CEF-4 (32).

The AP-1 mediated mechanism of the T64 transactivation is finally in agreement with the expression of clusterin in cells undergoing programmed cell death (14). Cell-suicide generally occurs through molecular processes also involved in cell division. Early phenotypic signs of the programmed cell death such as the loss of adhesion and the breakdown of the nuclear membrane are also observed during mitosis. In this respect, an abortive mitosis model of cell suicide has been proposed (26). Our work which extends the parallelism between the two dramatic cellular activities : death and proliferation, at the level of gene transcription factors, supports this new concept.

Moreover, it has recently been shown that the protein kinase C cascade is involved in neurodegeneration (48) and is altered in Alzheimer's disease (49), together with the dramatic induction of clusterin (18, 20, 21). An explanation of these observations could be provided by the present results, showing the determinant importance of the AP-1 site in the T64 induction. This result is also in excellent agreement with the finding that an accumulation of the c-*fos* protooncogene precedes the clusterin induction in involuting prostate (27). Results obtained so far in different cell death systems are contradictory about the role of proteine kinase C, suggesting a negative (50; 51) or a positive (48; 52; 53) involvement. The present work confirms that the proteine kinase C pathway plays a great part in the apoptosis program, but the nature, positive or negative, of its contribution in cell death is dependent on the precise influence, detrimental or protective, of the clusterin in this phenomenon.

Binding of nuclear factors to the PUR element correlates with the T64 promoter activity

The second regulatory region of the T64 transcription revealed by this work is a novel cis-acting element. We have termed this sequence PUR because of its homology with the so-called region originally identified in the human c-*myc* gene 5′-flanking region and also present in several other promoters (38). Although this previous work had focused attention on the possible involvement of PUR in replication origins, we cannot exclude that nuclear factors binding this domain in the c-*myc* and in the T64 promoters can be analogous. It is interesting to note that c-*myc* has also been implied in cell death (27; 54). Our observations, first that the PUR region is strictly required for T64 promoter activity, and second, that the intensity of the PUR DNA/nuclear protein complexes correlates with this activity, clearly demonstrate that

the PUR sequence is a central element involved in the T64 gene transcription. We have presented here two elements, AP-1 and PUR, necessary for the T64 promoter activation by v-*src*. It cannot be excluded however, that additional elements participate to this activation. This is particularly suggested by the loss of promoter activity caused by the removal of the −144 to −44 region carrying a CCAAT box and an AP-2 element. Work is in progress to further characterize the nature of the nuclear factors binding the PUR element, potentially involved in the apoptosis program.

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