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AP-1/jun binding sites mediate serum inducibility of the human vimentin promoter

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Abstract

The vimentin gene is inducible by serum in quiescent Balb/c 3T3 cells, but the molecular mechanism of this induction is unknown. The results presented here represent a first step towards the elucidation of the pathway of events leading from growth factor-receptor interaction to this induction. A series of Bal 31 deletions of the human vimentin promoter are used to show that a sequence residing at -700 is responsible for the serum, and also TPA inducibility of this gene. This sequence is able to confer serum inducibility upon uninducible constructs regardless of its position and orientation, indicating that it is this element alone which is required for this induction. The isolated sequence is a strong enhancer as well. Further deletions and the use of synthetic oligonucleotides demonstrate that a 24-mer containing two AP-1/jun binding sites confer both serum and TPA inducibility upon the human vimentin gene. Gel retardation analysis confirms that this sequence binds an AP-1 -like protein.

INTRODUCTION

Vimentin is a subunit of one class of intermediate filaments which play a major role in the cytoskeleton of the cell. The role of these filaments is poorly understood (1). The expression of vimentin is developmentally regulated, such that vimentin containing filaments occur in the adult in cells of mesenchymal origin. Vimentin is also expressed in most cultured cells regardless of their embryonic origin.

A vimentin cDNA was isolated as a growth regulated gene by differential screening of cDNA libraries (2). Subsequently it was shown that vimentin mRNA levels are inducible by growth factors in a variety of quiescent cell types (3,4,5). In quiescent Balb/c 3T3 cells, vimentin mRNA levels are inducible by PDGF but not by EGF or insulin(6), making this gene a member of the competence gene family. In tsAF8 cells, vimentin mRNA is inducible by serum in the presence of cycloheximide(7). In addition to peptide growth factors, vimentin mRNA and protein synthesis are inducible by phorbol esters in K562 cells(8) , HL-60 cells(6) and in mouse myeloma cells(9). In each of these cases, phorbol esters inhibit rather than stimulate cell proliferation. Thus vimentin mRNA is inducible by agents which both stimulate and inhibit cell proliferation.

There are two interesting aspects of any growth regulated gene: first, what is the role of the gene product in cell proliferation, and second what is the mechanism of the

induction of the gene. In the case of vimentin, the answer to the first question remains obscure. Vimentin monomers and filaments are phosphorylated by both protein kinase C and cAMP dependant protein kinases (10,11), and there is evidence that phosphorylation leads to vimentin disassembly (10). Vimentin filaments are reorganized during mitosis, and it has been suggested that phosphorylation may be involved in this process as well(12). However, while there are multiple correlations between intermediate filament processing and growth, the direct link is still poorly understood.

The present communication is concerned with the second question, i.e., what is the mechanism by which growth factors and phorbol esters activate vimentin gene expression. The vimentin promoter and 5' flanking DNA has been cloned and shown to confer PDGF inducibility to a linked CAT gene(13). Here, deletion analysis is used to delineate the sequences required for this effect. We have identified a 24 nucleotide sequence which mediates both serum and phorbol-12-myristate-13-acetate (TPA) inducibility. This sequence resides at -700, has strong enhancing ability, and contains two AP-1/jun binding sites. Gel retardation analysis confirms that this sequence binds nuclear proteins, and this binding can be competed by an oligomer containing a prototypical AP-1 binding site.

MATERIALS AND METHODS

Cells Balb/c 3T3 cells were grown in Dulbecco's modified eagle medium (MEM) with 4500 mg/l glucose, supplemented with glutamine, 10% calf serum and antibiotics, in a 10% CO_2 atmosphere. Cells were routinely made quiescent by plating at $5x10^3$ cells/cm^2 followed by growth to confluence (4 days). The medium was then replaced with complete medium containing 1% calf serum and the cells were used 3 days later. In every experiment, the degree of quiescence and stimulation of the cells was checked by autoradiography: quiescent cells typically had a labeling index of 2-5 % and stimulated cells 90%. If the values were significantly different from these, the results were not used. Transfection was by the suspension (14) method, or in some experiments in monolayer only using the same solutions and shock conditions as for the suspension method. Cells were transfected with a total of 10 ug DNA/ $3x10^5$ cells. This included 8 ug of test plasmid plus 2 ug of control or selectable marker plasmid. For transient transfection experiments, cells were cotransfected with pCH110(15) and harvested 48 hours later. Cell lysates were prepared by three cycles of freeze/thaw, and CAT activity assayed as described by Gorman et al (16). Spots were cut from the TLC plates and counted in a liquid scintillation counter. B-galactosidase activity was assayed as described(15) and CAT activities were expressed relative to the B-galactosidase activity.

Stable cell lines For G418 selection, cells were cotransfected with pRSVneo (17). Two days after transfection, the cells were replated in 6 well cluster dishes at $1.3x10^4$ cells/

well in 0.4 mg/ml G418 (Gibco). Cells from an individual transfection were plated in six wells. When the colonies were large enough to be seen without the microscope, each well was trypsinized to redistribute the cells in the well. When the cells were 90% confluent, all the cells from a single well were transferred to two 100 mm dishes. These cells were frozen when they just reached confluence. For analysis, the cells were thawed and passed twice before plating for an experiment.

Plasmids The starting material for all the constructs described was pG4F1EM1.5 (13) which contains 1400 nt of 5' flanking DNA and 73 nt of 5' untranslated region. This plasmid was linearized at the Apa 1 site at -860 and digested with Bal 31. Following digestion with BamH1, which cuts at the 3' end of the vimentin fragment in the polylinker, appropriately sized fragments were isolated by electrophoresis on polyacrylamide gels. These were cloned into pUCCAT (13) which contains a promoterless CAT gene. Plasmids were prepared by centrifugation over $CsCl_2$ gradients, and were sequenced by the method of Sanger (18) to determine the 5' endpoint.

Gel Retardation Assays Nuclear extracts were prepared from $5x10^7$ exponentially growing Balb/c 3T3 cells essentially by the method of Dignam et al (19), except that the nuclei were extracted with 0.8M KCl. The clarified extract was dialyzed vs. buffer D contining 0.2mM PMSF, 5 ug/ml antipain, and 5 ug/ml leupeptin, and stored at -100°C. Gel retardation assays were performed by mixing 20 ug nuclear extract protein with 4 ug poly (dI-dC)-poly (dI-dC) in binding buffer (10 mM Hepes, pH 7.9, 50 mM KCl, 10 mM EDTA, 5 mM DTT, 5 mM $MgCl_2$)with protease inhibitors. After 15 min at room temperature, competitor DNAs plus ^{32}P end labeled vimentin AP-1 oligomer (see figure 5 for sequence) were added. After another 15 min incubation, the complexes were electrophoresed over a 4% polyacrylamide gel (80:1 acrylamide:bis ratio) in 0.25x TBE. Following electrophoresis, the gel was transferred to 3MM paper and autoradiographed. Competitor DNAs were the vimentin 24mer and the human metallothionein (HMT IIA) AP-1 binding site CGTGACTCAGCGCGC (20) as specific competitors, and a 115 nt fragment of the human IGF-1 coding sequence as non-specific competitor.

RESULTS

Vimentin mRNA levels are increased in Go fibroblasts by serum stimulation, and although it has been shown that phorbol esters can induce vimentin mRNA levels in myeloid cells (6,7,9), this same effect has not been demonstrated in fibroblasts. Figure 1 shows a comparison of the effects of serum and TPA on cytoplasmic vimentin mRNA in quiescent Swiss 3T3 cells. 10 % calf serum clearly induces vimentin mRNA levels in these cells, as does 60 ng/ml TPA. The effect of this concentration of TPA is not as great as that of serum at this time point. Essentially identical results were obtained in Balb/c 3T3 cells.

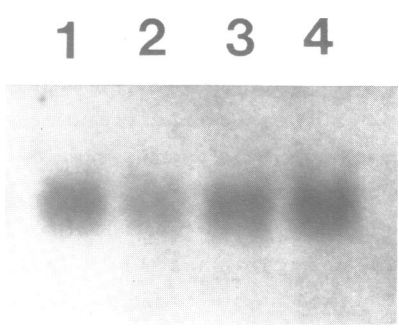

Figure 1
Vimentin mRNA levels in Swiss 3T3 cells. Swiss 3T3 cells were made quiescent as described in methods and treated with 10% calf serum or with 60 ng/ml TPA for eight hours. Cytoplasmic mRNA was isolated and used for Northern analysis. The blot was probed with the insert of the vimentin cDNA clone L7A3A (6). The section of the gel containing the vimentin band (2 kb) is shown. RNA was isolated from lane 1: exponentially growing cells , lane 2 : Go cells, lane 3: TPA stimulated cells, lane 4: serum stimulated cells.

Under these conditions, TPA is able to stimulate cell proliferation slightly (32% vs 75% for 10% serum).

In order to begin to address the mechanism of these responses, a series of 5' deletions in the vimentin promoter were made. These deleted promoter constructs all contain 73 nucleotides of 5' untranslated sequence, and are fused to a promoterless CAT gene (13). We have shown previously that the vimentin promoter contains two positive elements, one which maps between -830 and -529, the other between -241 and -150. In addition there is a negative element between -333 and -241 (13). In order to map these elements more precisely, a series of Bal 31 deletions of the vimentin promoter were made, starting from nt -860, as described in Methods. The mutant promoter fragments were cloned into the pUCCAT plasmid, and these constructs were transfected into exponentially growing Balb/c 3T3 cells together with the plasmid pCH110, which contains the B-galactosidase gene under the control of the SV40 early promoter (15). 48 hours after transfection, the cells were harvested, and lysates prepared and analyzed for CAT and B-galactosidase activity. CAT activity, expressed as % acetylated chloramphenicol, was normalized to B-galactosidase activity to correct for differences in transfection efficiency between the different plasmids. In each set of experiments, the normalized expression of each of the plasmids is expressed relative to one plasmid, in order to facilitate comparison between experiments. Figure 2 shows the results of two such series of experiments. The starred bars represent the results of one series of experiments, while the unstarred bars are another series. Each point represents the average of 2-4 determinations. In the experiment of the starred bars, the activity of the -425 deletion was arbitrarily set at 40 to fit with the

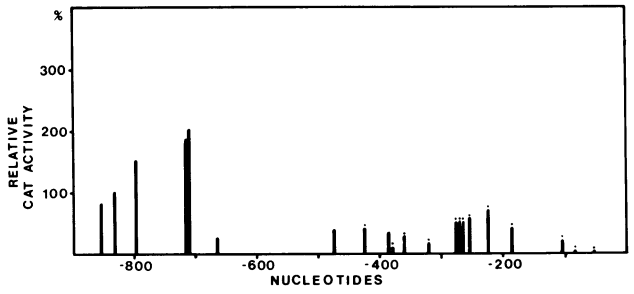

Figure 2

Transient transfection analysis of 5' deletions of the vimentin promoter. 5' deletions of the vimentin promoter were made by using Bal 31, and the resulting fragments were cloned upstream of a promoterless CAT gene. These plasmids were transfected into Balb/c 3T3 cells together with pCH110, containing the B-galactosidase gene, by calcium phosphate mediated transfection. After 48 hours the CAT activity of the cells was determined and normalized to the B-galactosidase activity to correct for differences in transfection efficiency. Two series of experiments are shown, one indicated by the starred bars, and one by the unstarred bars. Within each series the results are expressed relative to the normalized CAT activity of an arbitrarily selected plasmid. In the experiment indicated by the stars, the activity of the -425 deletion was set to 40 to facilitate comparison with the experiment indicated by the unstarred bars; all the starred points are expressed relative to this -425 deletion. Each point represents the average of 2-4 determinations.

data from the other experiment; all the other starred deletions were expressed relative to this construct.

The most striking result from this series of experiments was the strength of the positive element at -700. This data maps this element to a 48 nucleotide sequence between -713 and -664. In transient transfections, loss of this element decreases activity of the promoter by 5-8 fold. The negative element, whose removal approximately doubles CAT activity maps to positions -322 to -277. Another potential negative element is revealed by this analysis, mapping to -853 to -795, as removal of these sequences increases the CAT activity. This effect was seen in every experiment, but further work has not been performed to characterize this element. The boundaries of the proximal positive element are difficult to define from these results. It is apparent that its 5' end lies at -225. However, from -225 to -76, each removal of additional nucleotides results in a further decrease of the transcriptional activity of the promoter. Thus, it is likely that multiple elements having positive activity reside within the sequence from -225 to -76. Loss of activity in deleting sequences from -104 to -84 is likely to be due to loss of the CAAT homology which resides at -100. There are potential SP1 binding sites (21) at -140 and at -60, which may have a role as well.

These deleted promoter constructs were then used to determine the sequence responsible for serum inducibility. The vimentin-CAT constructs were cotransfected into Balb/c

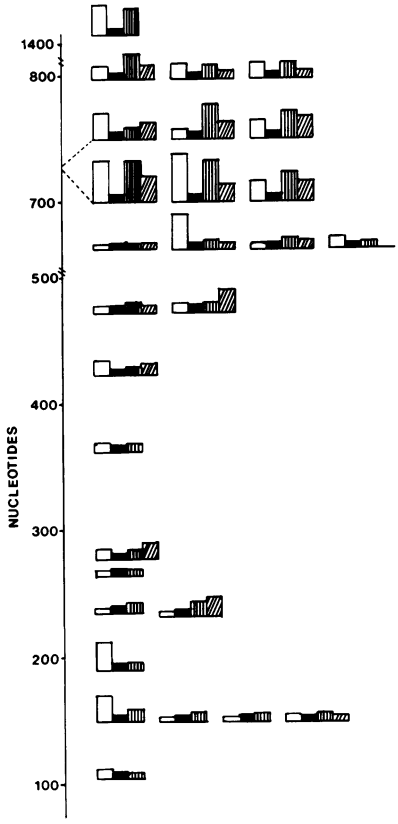

Figure 3
Serum and TPA inducibility of the deleted promoter mutants. The promoter deletion mutants described in Figure 2 were transfected into Balb/c 3T3 cells together with pRSVNeo, and G418 resistant colonies were selected. These colonies were expanded, and the CAT activity was determined under different conditions of growth. The cells were made quiescent and stimulated as described in Methods. After treatment, the cells were trypsinized and counted, and the CAT activity of equal numbers of cells was determined. Each group of three or four bars above represents the analysis of a single cell line. To facilitate comparison between cell lines, the CAT activity of the Go cells is set to 1.0, and that of the cells after treatment is expressed relative to the Go level. The position of the base of the bars relative to the scale on the left indicates the 5' endpoint of the deletion. In each case the open bars represent exponentially growing cells, the solid bar Go cells, the vertical stripes cells stimulated with 10% serum, and the diagonally striped bars cells treated with 60 ng/ml TPA. Multiple cell lines containing the same plasmid were analyzed in most cases.

3T3 cells with pRSVneo, and cells were selected with 0.4 mg/ml G418. The selection protocol used results in pooled clones, and six such pooled clones were obtained for each construct. Two or three of these cell lines were analyzed for serum inducibility of CAT

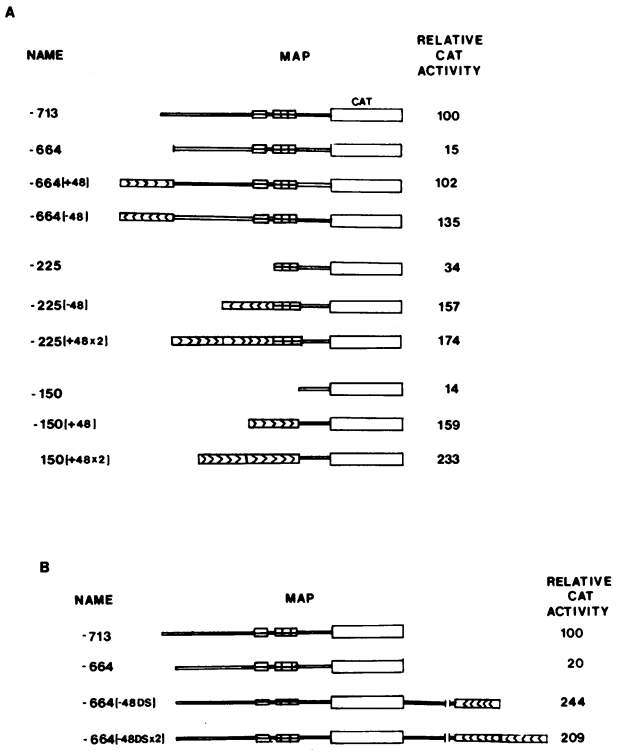

Figure 4

Transient transfection analysis of position and orientation mutants. The 48 nucleotide sequence required for serum and TPA inducibility was isolated from the -713 deletion by Hind III-Ava I digestion and cloned into various locations in the vimentin promoter in both orientations. On the left is the name of the construct. A map of each construct is also shown. The open box containing arrowheads indicates the 48 nt element, with the direction of the arrowheads indicating its orientation. The thin lines indicate vimentin sequences. The negative element is indicated by the open boxes with a horizontal line, the proximal positive element by the crosshatched box. The vimentin sequences are drawn to scale, while the 48 nt element and the CAT sequences (open box) are not. On the right is shown the relative CAT activity obtained when these constructs are analyzed by transient transfection in Balb/c 3T3 cells. These values are normalized to B-galactosidase activity and are expressed relative to the activity of the -713 deletion. Each number represents the average of two-three determinations. A: upstream constructs; B: downstream constructs.

enzyme. Cells from the different cell lines were plated in 60 mm dishes and grown for three days, at which point exponentially growing cells were harvested. One day later, the remaining dishes were shifted to 1% calf serum and incubated for 72 hours, then the cell were treated for eight hours with either 10 % calf serum or 60 ng/ml TPA. The cells

TABLE 1: Serum and TPA inducibility of position and orientation mutants. The mutants described in Figure 4 were transfected into Balb/c 3T3 cells and stable cell lines were selected and analyzed as described in methods. Results from a representative cell line are shown; in each case two or three cell lines were examined with essentially the same results.

CONSTRUCT NAME	RELATIVE CAT ACTIVITY IN EXP	GO	CS	TPAa
-713	1.5	1.0	5.6	2.4
-664	2.0	1.0	1.1	0.8
-664[+48]	5.1	1.0	2.6	3.0
-664[-48]	3.1	1.0	2.7	2.6
-225	0.8	1.0	0.8	N.D.
-225[-48]	3.0	1.0	9.5	6.6
-225[+48X2]	4.8	1.0	4.3	3.8
-150	1.0	1.0	1.2	N.D.
-150[+48]	1.3	1.0	8.9	12.5
-150[+48X2]	2.3	1.0	3.0	2.8

[a] EXP = exponentially growing cells; GO = quiescent cells; CS = quiescent cells treated for eight hours with 10% calf serum; TPA = quiescent cells treated for eight hours with 60 ng/ml TPA.

were then harvested by trypsinization, counted, and equal numbers of cells assayed for CAT activity. Each cell line has different absolute amounts of CAT activity, which roughly parallels the activity of the transfected plasmid in transient experiments. However, the number of copies of plasmid in these cell lines has not been quantified, so comparisons between different lines are only approximate. Therefore, in order to compare the serum and TPA inducibility of the different cell lines CAT activity in each individual cell line is expressed relative to the Go value, which is set at 1.0. Figure 3 shows the results of analysis of many of these cell lines. In this figure, each group of bars represents the relative CAT activity in an individual cell line. The open bar is CAT activity in exponentially growing cells, the filled bar Go cells, the vertically striped bar serum stimulated cells, and the diagonally striped bar TPA treated cells. Each group of bars is arranged vertically according to the 5' end of its deletion corresponding to the scale on the left. For clarity, the deletions at -713 and -712 are separated. The criteria for serum inducib-

ility are 1) that the CAT activity in both exponentially growing and in serum stimulated cells be >2-fold higher than that in quiescent cells, and 2), that different cell lines with the same deletion show a similar pattern. It is quite clear from this analysis that deleted promoters containing the positive element at -700 (DPE, or distal positive element) retain both serum and TPA inducibility. No constructs lacking this element can confer this inducibility. While there is an occasional construct which meets the first criteria stated above, such as the first deletion at -150, in no case are both the criteria met if the DPE is not present. TPA inducibility follows serum inducibility, and appears to be mediated through the DPE as well.

Next, we asked whether the DPE was both necessary and sufficient for serum inducibility, or if it required participation of other elements in the promoter. To answer this question, a 48 nucleotide fragment extending from the HindIII site of the -713 construct to an Ava 1 site at -666 was isolated. This fragment was then cloned at the 5' end of various deletions, thus effectively creating internally deleted promoter constructs. Thus the 48 nt fragment was cloned at the 5' end of the -225 deletion, thus eliminating the negative element, and at the 5' end of the -150 deletion, eliminating both the negative and some of the proximal positive element. This fragment was inserted in these positions in both orientations, and in single and double copies. This fragment has also been placed downstream of the CAT gene.

Transient transfection analysis was used to assess whether or not this element functions as an enhancer in this context. Figure 4 shows the results of two series of experiments in which the different constructs were transfected into Balb/c 3T3 cells, and relative CAT activity determined as described above. Again, each point represents the average of three determinations. The map of the different constructs is shown, showing the DPE, as well as the negative and proximal positive elements. The results show that the DPE has strong positive activity regardless of its position and orientation in the promoter. The effects of the three elements can be seen in the parental constructs (-713, -664, -225, and -150). Reinsertion of the 48 nt fragment at its original location in the right orientation restores wild type activity (compare -713 and -664(+48)), as it does in the opposite orientation. High activity is also observed when the negative element is eliminated in the -225 series. However, the positive effect is not significantly greater than when the negative element is present, implying that the DPE is able to override the effects of the negative element. Insertion of two copies of the DPE at -225 leads to slightly higher CAT activity, but much less than two-fold higher. Again, the -150 series shows that the proximal positive elements have little effect. In this position, insertion of two copies of the DPE results in significantly increased CAT activity, but still not to the level of a two-fold increase. Figure 4B shows the results of a similar experiment with the "downstream" constructs. These constructs are based on the -664 deletion. Again

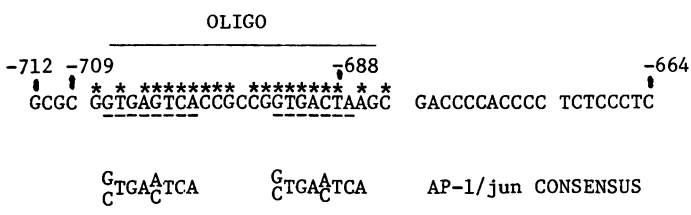

Figure 5
Sequence of the 48 nucleotide element. AP1/jun binding sites are underlined; the region of dyad symmetry is indicated by asterisks. The Sp1 binding site is indicated by dashed lines, and the oligonucleotide sequence is indicated.

the DPE confers strong positive activity, increasing the CAT activity 10-fold over the parental plasmid. In this case there is not a significant difference between the plasmids containing one and two copies of the DPE.

The serum inducibility of these constructs was analyzed by using cell lines as described above. The results of this analysis are shown in Table 1. Again, these results represent CAT activity in equal numbers of cells, expressed relative to the value for Go cells. It is clear from this analysis that the DPE confers serum inducibility to uninducible constructs regardless of its position and orientation. In fact, it seems that the magnitude of induction is often greater with constructs lacking the negative and positive elements. Again, the orientation of the DPE has no effect on its ability to mediate serum and TPA inducibility. Constructs which contain two copies of the DPE show lowered inducibility compared to a single copy in the position in the promoter, but show a similar level of inducibility to the wild type promoter (compare -150(+48), -150(+48x2), and -712). Possibily, this represents a decreased sensitivity of these constructs, which may be due to elevated promoter activity in quiescent cells. Taken together, these results and those of Fig 4 indicate that the vimentin DPE represents a serum inducible enhancer, as is the case with the fos gene (22, 23).

Figure 5 shows the sequence of the 48 nucleotide element. The most striking feature of the sequence is the presence of two AP-1/jun binding sites separated by five nucleotides (indicated by the dashed line). There is as well a perfect dyad symmetry about the central GC of these five nucleotides, as indicated by the asterisks. These elements occupy only 24 nucleotides of the 48, however, and we wanted to know whether the other nucleotides played a role in the serum inducible enhancing activity. Of particular interest was an Sp1 binding site (21) at the extreme 5' end of this sequence (indicated by the dashed lines). To address these questions two 5' deletions of the 48 nucleotide element were prepared, as well as an oligonucleotide containing only the 24 nucleotides comprising the two AP1/jun binding sites. The positions of the 5' end of these deletions, -709 and -688,

TABLE 2: Transient activity and serum inducibility of mutants of the 48 nucleotide region. Cells were transfected and analyzed as described in Methods.

CONSTRUCT NAME	Relative CAT Activity In: TRANSIENT ASSAY	STABLE CELL LINES EXP	GO	CS	TPA[a]
-713	100	2.8	1.0	4.3	3.4
-664	25	0.8	1.0	1.2	0.9
-709	216	3.0	1.0	5.8	3.2
-688	13	1.1	1.0	0.5	0.8
Oligo (+)	142	9.6	1.0	4.7	2.6
Oligo (-)	208	2.0	1.0	1.5	1.8

[a] EXP = exponentially growing cells; GO = quiescent cells; CS = quiescent cells treated for eight hours with 10% calf serum; TPA = quiescent cells treated for eight hours with 60 ng/ml TPA.

are indicated in this figure, as well as the sequence of the oligonucleotide, which was inserted into the -664 construct in both the correct (+) and the reverse (-) orientations.

Table 2 shows a summary of the results obtained using these constructs. Included in this table are the results of both the transient assays, and of the cell lines. Whenever the 24 nucleotide sequence used for the oligonucleotide is included, as in the -709, oligo (+), and oligo (-), both high levels of expression in the transient assay and serum and TPA inducibility are observed. The -688 construct behaves similarly to the -664 parental plasmid: there is low activity in the transient assay, and the associated CAT activity is not serum inducible. This experiment shows that only these 24 nucleotides comprise the serum inducible enhancer; neither the Sp1 binding site, nor the sequence from downstream from -690 are required for these effects.

In order to confirm that this sequence actually binds an AP-1 like complex, gel retardation assays were performed using nuclear extracts prepared from exponentially growing Balb/c 3T3 cells. Proteins extracted from these nuclei in 0.8M KCl formed a complex with the vimentin oligomer, resulting in at least two retarded bands following electrophoresis (Figure 6, lanes 1 and 2). These bands are specifically competed by both cold vimentin oligomer and by a protypical AP-1 binding site (HMT IIA(20)), but not by an unrelated oligomer. Moreover, the kinetics of competition by the vimentin oligomer and the HMT IIA oligomer are indistinguishable, indicating that the proteins which interact with these two sequences are similar.

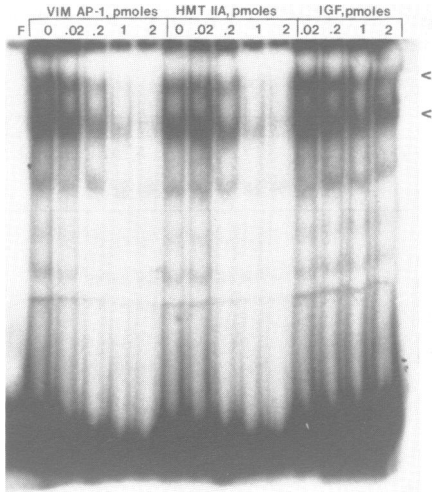

Figure 6

Binding of nuclear proteins to the vimentin AP-1 oligomer. Nuclear proteins were extracted from exponentially growing Balb/c 3T3 cells with 0.8M KCl, and gel reardation assays were performed as described in Methods. The probe was the double stranded vimentin oligomer described in Figure 5, ^{32}P labelled at both ends with T4 polynucleotide kinase to a specific activity of 10^9 dpm/ug. Each reaction mixture contained .006 pmoles of probe and competitor DNA. Above each group of lanes is indicated the competitor DNA used, and above each lane is shown the number of pmoles of competitor DNA. These amounts of competitor DNAs give 3,30,367,and 300 fold excess of cold DNA over hot probe. The competitor DNAs (see Methods) are derived from the vimentin serum response element (VIM AP-1), the human metallothionin IIa promoter (HMT IIA), or the IGF-1 coding sequence (IGF-1). F, probe alone without nuclear extract. Arrows indicate the positions of the major retarded complexes.

DISCUSSION

The results presented here show that a 24 nucleotide sequence containing two AP1/jun binding sites is both necessary and sufficient to confer serum inducibility upon the vimentin promoter. This sequence resides at -700 in the vimentin promoter, which is surprisingly far upstream. Since this sequence stimulates serum inducibility when it is moved as close as -150, the reason for the great distance of this element from the cap site is unclear. This 24 nucleotide sequence also functions as a strong enhancer, as has been shown for a single AP-1 site in other contexts (24,25). The function of the other elements in the promoter which have been identified by transient transfection is as well not clear from these experiments; perhaps they are required for developmental control of vimentin gene expression.

There are many correlates between AP-1/jun and growth. First, AP-1 is the cellular

homolog of the v-jun oncogene (26,27). AP-1 mediates the transcriptional stimulation of a variety of genes by TPA (24,25).However, the data presented here represent the first direct demonstration that these binding sites can mediate serum and presumably growth factor inducibility as well. The use of stable cell lines was critical to this work, since the magnitude of the induction of the vimentin gene by serum in transient transfection assays was always around two fold. This is probably because a "deep" quiescence is required for vimentin gene transcription to be shut off, which is not the case for other growth factor regulated genes such as c-fos. It has been shown that the binding activity of PEA-1, which is probably the mouse homolog of AP-1, is serum inducible (28), and it is possible that it is this binding activity which mediates vimentin induction.

What are the proteins which comprise this binding activity and mediate the response of the vimentin gene to serum? AP-1 is actually a complex of several proteins which includes c-jun (26,27), c-fos (29,30,31), and fos-related antigens(28).All of these proteins have been shown to be inducible by serum (32,33,34,35,36). Thus one possible mechanism by which the vimentin gene is induced may be through these proteins i.e., fos, jun, and possibly other proteins are induced by serum, a protein complex forms and binds to the vimentin 24 nucleotide sequence, activating transcription. Arguing against this possibility, however, are the observations that vimentin mRNA levels (7) and transcription are induced by serum in the presence of cycloheximide and anisomycin (manuscript in preparation), as is the case for c-fos and c-myc (37). These data suggest that de novo protein synthesis is not required for the induction of the vimentin gene by serum, which would rule out involvement of any proteins which are not present in quiescent cells. There are two additional lines of evidence which support this hypothesis. First, the transin gene which contains a prototypical TPA inducible AP-1 site in its promoter, is inducible by growth factors, but this induction is completely inhibited by cycloheximide (38,24). If, as appears likely, this AP-1/jun binding site mediates the growth factor induction, it does so by a completely different mechanism than that operating in the vimentin gene, and perhaps via fos/jun. And second, there is data indicating that the TPA induction mediated by AP-1 binding sites operates via a post-translational mechanism (25). Taken together these observations indicate that more than one kind of protein complex can bind to this class of binding sites: one containing proteins already present in quiescent cells, and one requiring de novo protein synthesis. Since there is very little fos protein in quiescent cells (39), this protein is unlikely to be involved in an induction event which does not require protein synthesis. jun mRNA, and presumably protein, are present in low but detectable amounts in quiescent cells (32,33) so perhaps this protein acts alone, or forms a complex with other, non growth regulated proteins. We are currently conducting experiments to determine the nature of the protein complex which binds to the vimentin 24 nucleotide sequence.

The presence of duplicated AP-1/jun binding sites in the vimentin promoter is unusual among cellular genes containing such sites, and may account for the strong serum inducibility of this element (as much as 10 fold). This arrangement of multiple protein binding sites is reminiscent of viral enhancers, and in fact this sequence bears a strong resemblance to the polyoma enhancer, which contains a single AP-1/jun site(28,40,41). There is a region of perfect dyad symmetry about the central C of the five nucleotide sequence which separates the two binding sites, so it appears that the two binding sites may function together as a single element. We are in the process of determining whether both binding sites are required for the function of this element.

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