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SV40 Large T Antigen with c-Jun Down-Regulates Myelin P_0 Gene Expression: A Mechanism for Papovaviral T Antigen-Mediated Demyelination

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Summary

Expression of myelin proteins has been shown to be altered in transgenic mice that express papovaviral large tumor (T) antigens. This paper analyzes the effect on P_0 gene expression in secondary Schwann cells transfected with the SV40 T antigen gene and in Schwann cells immortalized by T antigen. In secondary Schwann cells, both T antigen and c-Jun are required for significant inhibition of the P_0 promoter; expression of only one of the proteins is insufficient for repression of the P_0 gene. T antigen, c-Jun (p39), and c-Jun-related protein (p47) form an immunoprecipitable complex in SV40 immortalized Schwann cell lines, and T antigen and c-Jun bind independently and as a complex to the P_0 promoter. Our data suggest that the probable molecular mechanism underlying the hypomyelination observed in transgenic animals expressing T antigen may be due to the repression of the P_0 gene by T antigen and c-Jun.

Introduction

Schwann cells in the peripheral nervous system are responsible for formation and maintenance of myelin sheaths around axons. Breakdown of the myelin sheaths (demyelination) or defective formation of the myelin sheaths (dysmyelination) prevents propagation of electrical impulse along nerves and leads to neurological symptoms and signs. Neither the agents that cause demyelination nor the mechanisms whereby these agents produce demyelination are fully understood. It has been shown, however, that papovavirus tumor (T) antigen causes the human demyelinating disease, progressive multifocal leukoencephalopathy (Major et al., 1992), and transgenic animals expressing simian virus 40 (SV40) large T antigen develop an extensive peripheral neuropathy characterized by demyelination of large diameter axons (Messing et al., 1985; Dyer and Messing, 1989; Messing, 1990). SV40 large T antigen is a multifunctional phosphoprotein of apparent molecular weight 94,000 that is necessary for viral replication and cell transformation (for reviews see Fanning and Knippers, 1992; Fanning, 1992; Pipas, 1992). In an attempt to understand how T antigen alters myelination, we studied the synthesis of myelin proteins and the formation of myelin sheaths in Schwann cell lines immortalized by the SV40 T antigen gene (Tennekoon et al., 1987; Peden et al., 1989).

Schwann cell lines expressing T antigen were generated after transfection of plasmids containing the SV40 T antigen gene under the control of an inducible mouse metallothionein I promoter ($pMT_{wt}SV$; Tennekoon et al., 1987). We found that T antigen expression interfered with myelin formation in vitro. Although Schwann cells that expressed high levels of T antigen stopped dividing when they contacted neurites, synthesized a basal lamina, and segregated the neurites into smaller fasicles, they failed to form myelin sheaths around the neurites (Tennekoon et al., 1987). This suggested that the molecules involved in axon-Schwann cell interactions (i.e., the myelin-associated glycoprotein; Owens and Bunge, 1991) were expressed normally in this cell line, but those molecules involved in the formation of compact myelin (i.e., P_0 protein; Trapp et al., 1981) were not. Schwann cells generated with the wild-type metallothionein promoter-regulating T antigen had a 10-fold lower level of the mRNA for the major peripheral myelin protein P_0 (Greenfield et al., 1973; for review see Lemke, 1988) than that in untransfected secondary Schwann cells (Tennekoon et al., 1987). When a synthetic metallothionein promoter-regulating SV40 T antigen was used, the Schwann cell line obtained (MT_4H1) expressed lower levels of T antigen and morphologically resembled untransfected Schwann cells (Peden et al., 1989). In addition, MT_4H1 cells maintained in the absence of the inducer $ZnCl_2$ were able to form myelin sheaths in vitro, but induction of T antigen in MT_4H1 by $ZnCl_2$ resulted in a significant loss of P_0 protein and mRNA expression (Peden et al., 1990).

Although the mechanism whereby T antigen alters P_0 expression is not understood, there are several hypotheses that have been proposed to explain how T antigen can alter cellular function. A mechanism that is well characterized is the interaction of T antigen with cellular proteins such as the retinoblastoma gene product (RB; DeCaprio et al., 1988), p53 (Lane and Crawford, 1979; Linzer and Levine, 1979), and the transcriptional factor, AP2 (Mitchell et al., 1987). Another mechanism would be the direct binding of T antigen to the cellular genome to alter cellular gene expression (Lane et al., 1985; Pollwein et al., 1987; Gruss et al., 1988). Large T antigen recognizes and binds to the pentanucleotide sequence, GAGGC, within the early promoter of SV40 (Jessel et al., 1976; Tjian, 1978; Tegtmeyer et al., 1983; DeLucia et al., 1983), and identical binding sequences have been found in the mammalian genome (Lane et al., 1985; Pollwein et al., 1987; Gruss et al., 1988). Although it has been shown that T antigen can bind to these sequences in the mammalian genome, the biological consequences of this binding are not understood. Additionally, T antigen has been shown to induce the expression of other

transcriptional factors such as c-Jun (Wasylyk et al., 1988) and Sp1 (Saffer et al., 1990), which in turn can alter cellular function.

The role of c-Jun in Schwann cell differentiation is of interest because, when Schwann cells in culture are differentiated with the use of cyclic AMP analogs or by activation of adenylate cyclase with forskolin, c-*jun* mRNA levels decrease, and P_0 mRNA levels increase (Lemke and Chao, 1988). Moreover, when Schwann cells dedifferentiate after nerve transection, c-*jun* mRNA expression increases, whereas P_0 mRNA levels decline (Hengerer et al., 1990; Trapp et al., 1988). The transcriptional factor c-Jun belongs to the bZIP family of proteins, which have a characteristic leucine zipper dimerization domain (Landschulz et al., 1988) and bind to the DNA motif TGAG/CTCA (Bohmann et al., 1987; Lee et al., 1987; Chiu et al., 1988; Angel et al., 1988). However, isolation and sequence analysis of the P_0 promoter revealed that the 5′ flanking region did not contain any AP-1 consensus elements (Lemke et al., 1988), and cotransfection of a c-Jun expression plasmid, along with a plasmid containing the P_0 promoter regulating a reporter gene (pP_0cat), failed to show any changes in reporter activity (Monuki et al., 1990). These studies would indicate that there is no direct involvement of c-Jun in regulating the expression of the P_0 gene.

P_0 protein is a major protein in peripheral nervous system myelin, and its expression is restricted to Schwann cells (Poduslo et al., 1984). The protein is necessary for the formation of the myelin sheath, since the lack of expression of the protein leads to a failure of myelin formation (Owens and Boyd, 1991; Giese et al., 1992). As we had noted an inverse relationship between T antigen and P_0 expression, we investigated the mechanism whereby SV40 T antigen alters P_0 expression, both in secondary Schwann cells and in Schwann cell lines immortalized by T antigen. In this paper, we report that SV40 large T antigen, c-Jun (p39), and a c-Jun-related protein (p47) form an immunoprecipitable complex, and it is likely that the repression of P_0 expression is mediated by this complex.

Results

Large T Antigen and c-Jun Together Down-Regulate the Expression of the Myelin P_0 Promoter

The effect of c-Jun and SV40 large T antigen on transcription of the P_0 promoter was determined by cotransfection experiments in secondary rat Schwann cells and in the immortalized MT_4H1 Schwann cell line. When the plasmid containing 1 kb of the P_0 promoter upstream of the bacterial chloramphenicol acetyltransferase (*cat*) gene (pP_0cat) was transfected into MT_4H1 cells grown in the presence of $ZnCl_2$ and into secondary Schwann cells, Cat activity in the cell lines was 2- to 3-fold lower than in secondary Schwann cells (Table 1). Basal Cat levels in MT_4H1 cells grown in the absence of Zn^{2+}, however, were comparable with the levels obtained in secondary Schwann cells (data not shown). Secondary Schwann cells cotransfected with pP_0cat and pRSVc-jun did not show any significant alteration of Cat activity, even with pRSVc-jun 2- to 5-fold in excess of pP_0cat in the transfection mixture (data not shown). When pP_0cat was cotransfected with an SV40 large T antigen expression plasmid (pRSVtag) in secondary Schwann cells, there was a 15%–20% reduction in Cat activity. When both T antigen and c-Jun expression plasmids were cotransfected, however, a 3- to 4-fold reduction in Cat activity was observed (Table 1). In the MT_4H1 cell line, in which T antigen is regulated by a relatively weak synthetic metallothionein promoter, cotransfection of pP_0cat with either large T antigen or c-Jun expression plasmids decreased Cat activity by 5- to 10-fold relative to the basal expression of pP_0cat in secondary Schwann cells (Table 1). Since no change was observed in pRSVcat when cotransfected with either pRSVc-jun and/or pRSVtag (Table 1), the reduction in expression from the P_0 promoter cannot be explained by promoter competition. Thus, these results established that large T antigen and c-Jun cooperatively repress the P_0 promoter.

Table 1. T Antigen and c-Jun Cooperatively Repress P_0cat Activity

Plasmids	% Cat Activity Relative to 1 U of β-Galactosidase	
	MT_4H1 Schwann Cell Lines	Secondary Schwann Cells
pP_0cat	35 ± 3.5	100 ± 3.2
pP_0cat + pRSVc-jun	8 ± 4.0	98 ± 2.2
pP_0cat + pRSVtag	7 ± 7.3	78 ± 9.0
pP_0cat + pRSVtag + pRSVc-jun	5.5 ± 2.8	36 ± 6.0
pRSVcat + RSVc-jun + pRSVtag	NC	NC

Percent cat activity is the percentage of the value obtained for P_0cat activity expression in secondary Schwann cells where indicated. Two micrograms of pP_0cat were cotransfected with 10 μg of pRSVc-jun and/or pRSVtag. Transfection efficiencies were corrected by adding 3 μg of RSV β-galactosidase. The total amount of DNA transfected was always 20 μg adjusted with salmon sperm DNA. Data represent average of six separate experiments ± SEM.

SV40 Large T Antigen and c-Jun Form an Immunoprecipitable Complex

SV40 large T antigen has been shown to form complexes with several proteins. For example, interaction with p53 has been well studied, and it was shown that the p53–T antigen complex enhances binding of T antigen to site III on the SV40 promoter (Tack et al., 1989). Interaction with the transcription factor AP-2 has also been demonstrated (Mitchell et al., 1987), but although AP-1 and AP-4 alter transactivation of the SV40 late viral genes by T antigen (Mermod et al., 1988), no complexes between these two proteins and large T antigen have been demonstrated. A complex between T antigen and c-Jun might block the tran-

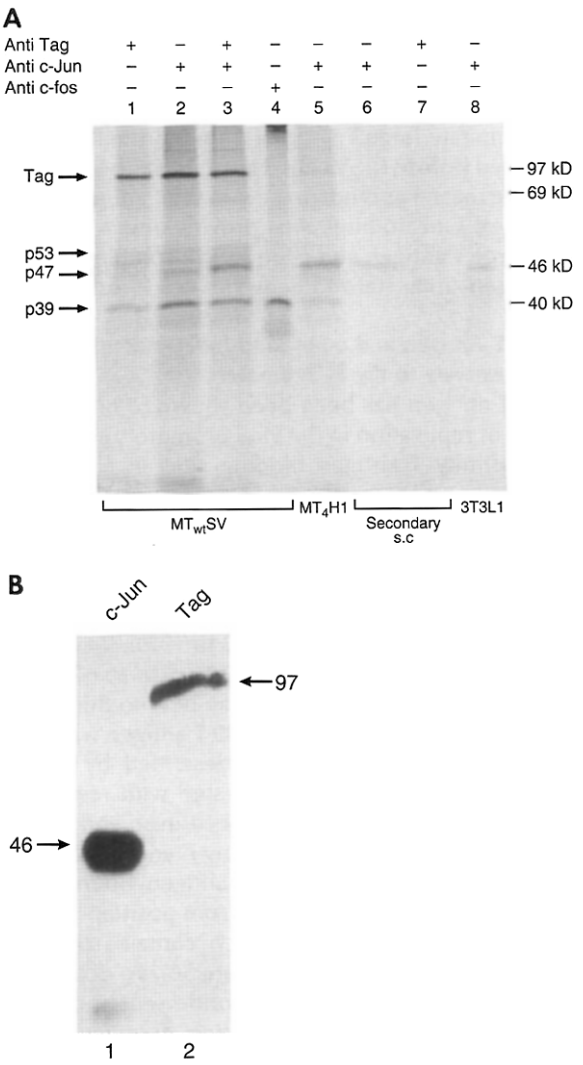

Figure 1. Large T Antigen, c-Jun, and c-Jun-Related Proteins Form a Complex

(A) Large T antigen and c-Jun form an immunoprecipitable complex. Immunoprecipitation of large T antigen and c-Jun in $MT_{wt}SV$ Schwann cells (lanes 1–4), MT_4H1 Schwann cells (lane 5), secondary Schwann cells (lanes 6 and 7), and 3T3 L1 cells (lane 8). The cell proteins were labeled with trans ^{35}S label in methionine- and cysteine-free medium, and nuclei were isolated according to Dignam et al. (1983). The proteins were immunoprecipitated with anti-large T antibody alone (lanes 1 and 7), with anti-c-Jun antibody (lanes 2, 5, 6, and 8), with a combination of anti-T antigen and anti-c-Jun antibodies (lane 3), and with anti-c-Fos antibody (lane 4). For each experiment, confluent dishes of secondary, immortalized Schwann cells or 3T3 cells were labeled with 0.5 mCi/ml trans ^{35}S label. The medium was free of L-methionine and L-cysteine and contained 5% dialyzed fetal bovine serum. The cells were labeled for 8 hr, and nuclei were isolated according to the method of Dignam et al. (1983) in the presence of 1% aprotinin and 0.3 mM phenylmethylsulfonyl fluoride.

(B) Immunoprecipitation followed by Western blot analysis of nuclear proteins from $MT_{wt}SV$ precipitated with anti-T antigen (lane 1) or anti-c-Jun antibody (lane 2). The immunoprecipitated proteins were analyzed on 10% SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose filters. The filters were probed with anti-c-Jun (lane 1) and with anti-T antigen (lane 2) antibodies by the ECL method for detection of antibody complexes.

scription of myelin P_0 if the complex bound to the P_0 promoter. To test this possibility, cellular proteins of MT_4H1 and $MT_{wt}SV$ (a cell line constructed with the wild-type metallothionein promoter regulating SV40 large T antigen; Tennekoon et al., 1987) were labeled with [^{35}S]methionine, and the respective nuclear proteins were isolated and then subjected to immunoprecipitation with either large T antigen or c-Jun antibodies. Antibodies directed against large T antigen resulted in precipitation of both T antigen (94 kd), c-Jun-related protein (45–47 kd; p47), and c-Jun (39kd; p39); in a similar manner with c-Jun antibodies, T antigen and c-Jun coprecipitated (Figure 1A, lanes 1–3 and 5). Confirmation that p47 was c-Jun-related protein was obtained by using serum-treated 3T3 cells (Figure 1A, lane 8), secondary Schwann cells (Figure 1A, lane 6), and HeLa cells (data not shown) as controls.

In a two-step procedure, we immunoprecipitated unlabeled nuclear extracts from $MT_{wt}SV$ and secondary Schwann cells with either anti-T antigen or anti-c-Jun antibodies and then analyzed the pellet on a Western blot. Nuclear protein extracts from $MT_{wt}SV$ cells immunoprecipitated with anti-T antigen showed the presence of only the p47 (Figure 1B, lane 1), but not protein extracts from secondary Schwann cells. Conversely, nuclear protein extracts from $MT_{wt}SV$ cells precipitated with anti-c-Jun antibodies showed the presence of T antigen on the Western blots (Figure 1B, lane 2), but not protein extracts from secondary Schwann cells. Addition of only the secondary antibody (anti-rabbit IgG) did not result in the detection of either protein. Furthermore, we were unable to detect c-Jun by using antibodies against p53 (PAb421; Harlow et al., 1981) for immunoprecipitation, indicating the specificity of the interactions (data not shown). We have also observed that T antigen and c-Jun form a complex in two other cell lines expressing SV40 T antigen (rat 3T3 and human fetal glial cells obtained from R. Frisque; unpublished data), indicating that the present results were not cell type specific. Moreover, in T antigen-expressing cell lines, antibodies to c-Jun and to large T antigen also immunoprecipitated a protein of 38–40 kd (Figure 1A, lanes 1–3 and 5). This protein p39 is a product of the c-Jun gene, and it has been shown to associate with the c-Fos protein (Rauscher et al., 1988). In HeLa cells, however, several polypeptides between 40–47 kd are immunoprecipitated with anti-c-Jun antibodies, and in these immunoprecipitates, the major protein band is between 45–47 kd. We find a similar result in secondary Schwann cells (Figure 1A, lane 6) and serum-treated 3T3 cells. In SV40-transfected Schwann cells, there is a marked induction of the p39 band (Figure 1A, lane 1), and in parallel cultures, there is an induction of both the 2.5 and 3.3 kb c-*jun* mRNA (data not shown). The p47 protein from HeLa cells has sequence homology with c-Jun (Bohmann et al., 1987) as well as AP-1-binding activity (Lee et al., 1987), and we refer to this protein as a c-Jun-related protein (p47). We also observed the association of the p39 protein with c-Fos protein when c-Fos

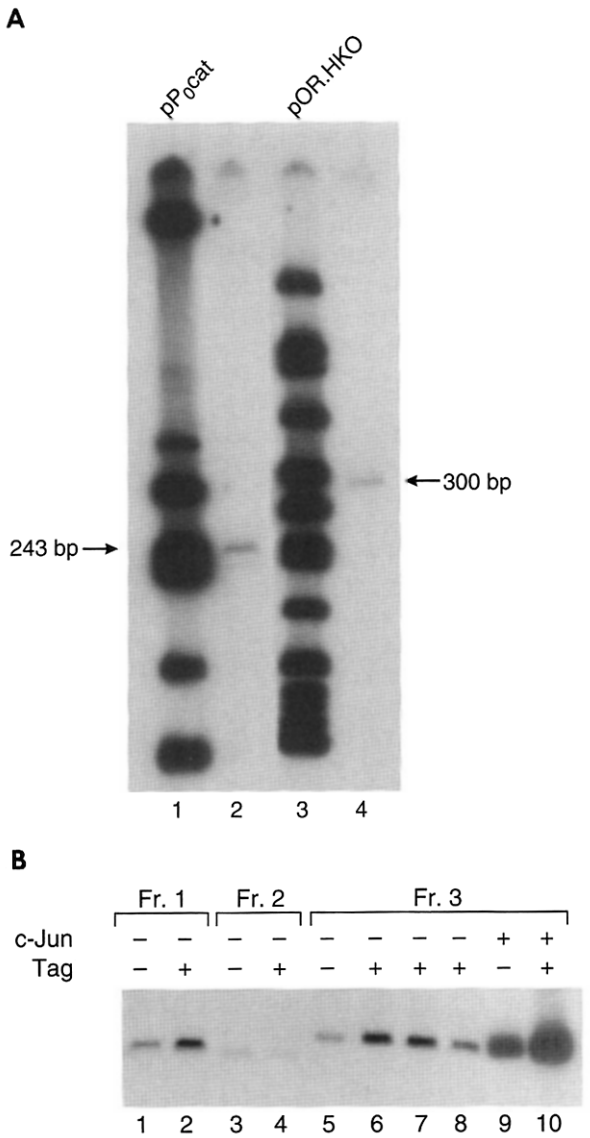

Figure 2. Large T Antigen and c-Jun Bind to the P_0 Promoter Protein–DNA binding immunoassay.

(A) The pP_0cat plasmid (Lemke et al., 1988) was digested with AccI, XbaI, and BglII (lane 1), and the pOR.HKO (Li et al., 1986) containing the SV40 origin of replication was digested with BstNI (lane 2). Then, 1–2 ng of labeled fragments (for details see Experimental Procedures) were incubated with 10–20 ng of purified large T antigen protein. The fragment bound by large T antigen was precipitated by the addition of 2 μl of monoclonal antibody PAb 419 and protein A Sepharose. The 243 bp fragment bound by large T antigen is from position −452 to −209 (lane 1).

(B) Isolated fragments were generated by PCR by using oligonucleotides (for details see Experimental Procedures) and immunoassay performed as above. Fragments 1 (from position −506 to −257) and 2 (from position −378 to −186) contain two of the pentameric sequences, and fragment 3 (from position −506 to −186) contains all three pentameric sequences. Fragment 1 (Fr. 1) is represented in lanes 1 (no T antigen) and 2 (200 ng of T antigen). Lanes 3 (no T antigen) and 4 (200 ng of T antigen) show lack of T antigen binding to fragment 2 (Fr. 2). Lanes 5–10 show binding to fragment 3 (Fr. 3) and had the following additions: lane 5, no added T antigen; lane 6, 10 μg of nuclear extract from $MT_{wt}SV$; lane 7, 200 ng of T antigen; lane 8, 200 ng of T antigen and 1.5 μg of unlabeled SV40 DNA containing T antigen–binding sites; lane 9, 20 ng of c-Jun protein; lane 10, 200 ng of T antigen and 20 ng of c-Jun.

antibodies were used for immunoprecipitation of proteins from serum-treated 3T3 cells, $MT_{wt}SV$, MT_4H1 (Figure 1A, lane 4), and secondary Schwann cells. However, antibodies directed against c-Fos did not coprecipitate large T antigen from a protein mixture obtained from $MT_{wt}SV$ cells (Figure 1A, lane 4), demonstrating that c-Fos is not present in the T antigen–c-Jun complex. Our findings thus provide direct evidence that large T antigen and c-Jun form a complex in SV40 T antigen–immortalized Schwann cell lines.

Large T Antigen and c-Jun Bind Independently and Cooperatively to the P_0 Promoter

SV40 T antigen has been been shown to bind to the origin of replication in the viral promoter region. The high affinity T antigen binding sites in SV40 DNA contain two pentanucleotide 5′-GCCTC-3′ sequences (Tjian, 1978; Tegtmeyer et al., 1983) separated by a 7 bp spacer (Ryder et al., 1985). Examination of the P_0 promoter region revealed four T antigen-binding pentameric sequences (see Figure 5); 5′-GCCTC-3′ (−735 to −731), 5′-GCCTC-3′(−383 to −379), 5′-GAGGC-3′ (−354 to −350), and 5′-GCCTC-3′ (−254 to −250). To investigate whether T antigen can bind to the P_0 promoter, baculovirus-purified SV40 T antigen was used in the immunobinding assay described by McKay (1981). pP_0cat plasmid was digested with restriction enzymes (see Experimental Procedures), and the recessed ends were labeled by using Klenow DNA polymerase and [α-^{32}P]dATP. The T antigen–bound DNA fragment was a 243 bp region (from position −452 to −209; Figure 2A, lane 1), which contains three of the pentameric sequences (Figure 5B). To determine which of the three pentanucleotide sequences was responsible for T antigen binding, fragments containing either two (fragment 1 from position −506 to −257 and fragment 2 from position −376 to −186) or all three (fragment 3 from position −506 to −186) elements were generated by polymerase chain reaction (PCR) (see Experimental Procedures for details), and immunobinding assays were performed. Purified T antigen showed binding to fragment 1 (Figure 2B, lane 2) and fragment 3 (Figure 2B, lane 6), but no binding was seen to fragment 2 (Figure 2B, lane 4). When a 50 M excess of unlabeled SV40 DNA containing the T antigen binding sites was added to the mixture, the P_0 promoter–T antigen complex in fragment 3 was markedly reduced (Figure 2B, lane 8). These results establish that SV40 large T antigen was able to bind to the P_0 promoter.

Although the P_0 promoter has no consensus AP-1-binding sites, c-Jun has been reported to bind to sequences that differ from the consensus element (Jones et al., 1988; Spandidos et al., 1989; Timmers et al., 1990). Since c-Jun in concert with SV40 T antigen repressed expression from this promoter, we tested whether c-Jun could bind to the P_0 promoter, presumably by using other sequence motifs. Immunobinding assays were performed with the isolated fragments as described for T antigen binding. Fragment 2 (data

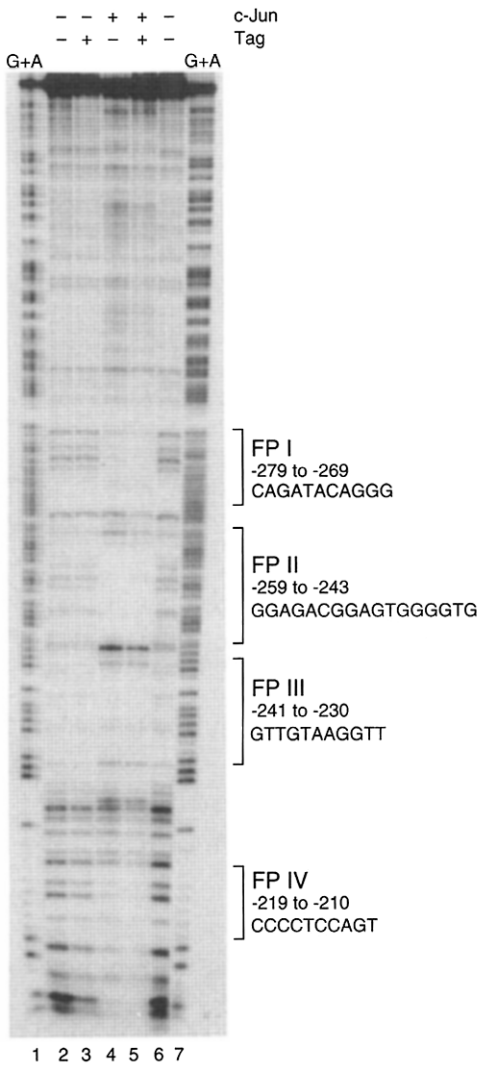

Figure 3. Large T Antigen Does Not Alter c-Jun Binding to the AP-1 Element on the P_0 Promoter

DNAase I footprint analyses. A PCR-generated fragment (from position −506 to −186) was end labeled on the antisense strand. DNAase I digestions were performed in the presence of T antigen (+Tag), c-Jun (+c-Jun), both (+Tag and +c-Jun), or in the absence of protein (−). Lanes 2 and 6 represent DNAase I digestions in the absence of protein with 0.1 or 0.2 U of DNAase I, respectively. Lane 3 represents DNAase I digestions in the presence of 1.3 μg of purified T antigen protein. Lane 4 represents DNAase I digestions in the presence of 10 ng of purified c-Jun protein. Lane 5 represents DNAase I digestions in the presence of both T antigen (800 ng) and c-Jun (10 ng). Sequencing ladders (for details see Experimental Procedures) are shown in lanes 1 and 7 (G + A).

not shown) and fragment 3 (Figure 2B, lane 9) bound to c-Jun, whereas fragment 1 showed no binding (data not shown). When both c-Jun and large T antigen were added to fragment 3, a significantly larger amount of the probe remained bound to the complex (Figure 2B, lane 10). These results show that there are additional sequence elements with which c-Jun can interact, and at least one of these is present in the P_0 promoter from −506 to −186.

To determine specifically the regions bound by c-Jun (AP-1) within fragment 3 of the P_0 promoter that contained all three T antigen-binding sites, DNAase I footprinting assays were performed (Figure 3). Reactions carried out with fragment 3 end labeled on the sense or the antisense strand indicated that c-Jun significantly protects one region of the P_0 promoter from position −259 to −243 (FP II; Figure 3, lane 4), which also overlaps a T antigen binding site (see Figure 5). Surprisingly, c-Jun also binds with lower affinity at three other sites on the P_0 promoter. The protection pattern on FP I (from position −279 to −269) is characterized by a small region that revealed enhanced bands, as well as few bands which were repressed in the presence of c-Jun, whereas FP III (from position −241 to −230) and FP IV (from position −219 to −210) display detectable hypersensitive bands throughout these regions. In agreement with Lemke et al. (1988), analysis of these regions did not reveal any apparent similarity to the reported AP-1 consensus sequence TGAG/CTCA (Lee et al., 1987; Chiu et al., 1988; Angel et al., 1988).

Since the T antigen-AP-2 complex had been shown to alter the ability of AP-2 to recognize its DNA-binding elements on the SV40 promoter and the human metallothionein promoter (Mitchell et al., 1987), we tested whether the addition of T antigen in the footprinting analysis would alter the properties of c-Jun binding. We found that when both T antigen and c-Jun were added to the mixture (Figure 3, lane 5), c-Jun continued to protect the high affinity binding site (FP II; from position −259 to −244) and the lower affinity binding site FP I (from position −279 to −269). Interestingly, addition of T antigen also increased the affinity of FP III (from position −241 to −230) and FP IV (position −219 to −210), such that bands throughout this region were weaker than when only c-Jun was added (Figure 3, lane 5). However, when T antigen was used by itself in a footprinting assay, we were unable to detect any significant protection in FP III or IV (Figure 3, lane 3). It is likely that the T antigen binding sites on the P_0 promoter are low affinity binding sites which cannot be detected by DNAase I protection.

Large T Antigen and c-Jun Inhibit P_0 Expression at a Transcriptional Level

To ascertain whether repression of expression from the P_0 promoter by SV40 T antigen and c-Jun is transcriptionally mediated, in vitro transcription assays were performed. Since the cotransfection experiments indicated that c-Jun alone did not alter pP_0cat activity, HeLa cell nuclear extracts, which contain c-Jun (Lee et al., 1987), were thought to be suitable for use. When various amounts of purified T antigen (from 10, 50, 100, 150, and 200 ng) were added to the nuclear extracts, a dose-dependent inhibition of transcription from the P_0 promoter was seen, with 50 ng of protein (molar ratio of protein to pP_0cat, 5:1) causing a 2-fold reduction (Figure 4, lane 4) and 200 ng of T

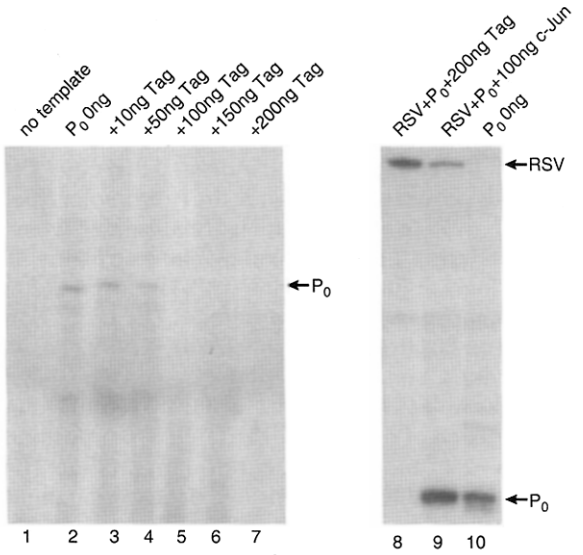

Figure 4. T Antigen and c-Jun Cooperatively Repress the P_0 Promoter at a Transcriptional Level

For in vitro transcription analyses of the P_0 promoter, reactions were performed with 8 μg of HeLa cell nuclear extract (Promega), along with 400 ng of pP_0cat. Reverse transcription and primer extension were performed as described in Experimental Procedures. Amounts (nanograms) of factors used are noted above each lane. Lanes 8 and 9 contain 400 ng of RSVcat template used as an internal control. Tag, T antigen.

antigen (molar ratio, 20:1) resulting in total repression (Figure 4, lane 7). Since there was no effect on expression from the RSV promoter when pRSVcat was used as an internal control, even in the presence of 200 ng of T antigen (Figure 4, lane 8), the T antigen–mediated repression of transcription of the P_0 promoter is specific and is not a consequence of increased amounts of protein. The molar ratio of template to factor required for in vitro repression of P_0 promoter by T antigen is similar to that observed for T antigen repression of the SV40 early promoter (Hansen et al., 1981). When 100 ng of c-Jun alone was added to HeLa nuclear extracts, there was no change in transcription from either the P_0 or RSV promoters (Figure 4, lane 9). These results indicated that the repression of the P_0 promoter required both T antigen and c-Jun and that this repression was transcriptionally mediated.

Discussion

This study was designed to investigate the molecular mechanisms governing the down-regulation of myelin P_0 gene expression found in SV40 large T antigen–transformed Schwann cells. Transient cotransfection of secondary Schwann cells with the T antigen and c-Jun expression plasmids pRSVtag and pRSVc-jun, respectively, and pP_0cat resulted in a marked repression of Cat activity. Transfection of only pRSVtag into secondary Schwann cells resulted in a moderate inhibition of pP_0cat expression, whereas transfection of pRSVc-jun had no effect (Table 1). We conclude that both T antigen and c-Jun are necessary to mediate the repression of the P_0 promoter and that the reason for the partial effectiveness of T antigen alone is most likely because secondary Schwann cells in culture themselves express some c-Jun (Figure 1, lane 6). In the T antigen–expressing MT_4H1 Schwann cell line, however, cotransfection of either c-Jun or T antigen expression plasmids and pP_0cat further repressed the P_0 promoter (Table 1). Presumably, this was due to MT_4H1 Schwann cells expressing higher levels of c-Jun than secondary Schwann cells (unpublished data). This is similar to SV40-transformed NIH-3T3 cells, in which transformation led to an increase in the level of c-Jun (Wasylyk et al., 1988). Cotransfection of other bZIP family proteins, such as c-Fos, Fra-1, Jun-B, or Jun-D, along with T antigen did not alter pP_0cat activity in secondary Schwann cells, indicating that the T antigen repression of the P_0 promoter is specific for c-Jun (data not shown). In vitro transcription assays using HeLa cell nuclear extracts, which contain c-Jun (Lee et al., 1987), showed that there was complete repression of transcription from the P_0 promoter upon addition of baculovirus-expressed T antigen, but no repression was observed following the addition of recombinant c-Jun protein alone (Figure 4). Thus, taken together, these results demonstrate that both c-Jun and T antigen are necessary for the down-regulation of myelin P_0 gene expression and that these proteins cause repression by acting at the transcriptional level.

Among the various hypotheses by which T antigen and c-Jun together could block P_0 transcription, direct complex formation between the two proteins with subsequent binding of the complex to the P_0 promoter or the independent binding of the two proteins to the DNA elements of the P_0 promoter seemed the two most likely possibilities. We have demonstrated that a complex between the two proteins exists, because by using monoclonal antibodies to T antigen, we were able to immunoprecipitate c-Jun and a c-Jun–related protein from extracts of $MT_{wt}SV$ and MT_4H1 cells (Figure 1A, lane 1); conversely, with antibodies to c-Jun, we were able to precipitate T antigen (Figure 1A, lanes 2 and 5). This complex is specific since the presence of T antigen was required to immunoprecipitate c-Jun and related proteins with an anti–T antigen monoclonal antibody, and the anti–T antigen monoclonal antibody did not immunoprecipitate c-Jun or related proteins from extracts of secondary Schwann cells (Figure 1A, lane 7). Therefore, T antigen, c-Jun, and c-Jun–related proteins form an immunoprecipitable intracellular complex. A similar complex has been observed in two other cell lines transformed by SV40 T antigen (rat 3T3 and human fetal glial cells; unpublished data), indicating that the formation of a complex between T antigen and c-Jun is a more general phenomenon and is not cell type specific.

Both SV40 large T antigen and c-Jun have been shown to complex with several cellular proteins. T antigen is found associated with the cellular phospho-

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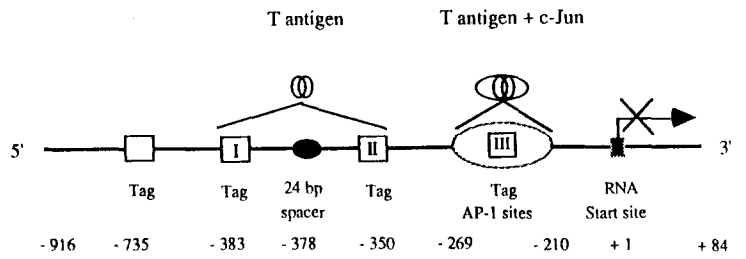

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ttaaactgctgcatggcaaaaggaatcgaaggatttcttaacagaagtgggggggggggagatctgggcttctt

cctggaagtttcctgatagagaaaatcttctgcctgggtagaatctcccaggatgcagggagatggaaaaagtt

gttccccagaggactttgtaGTCTACAGTGTTGTCGTAGCCATCGGAACAACGAGACACC

CTAATTTGGGAGTGCTCTGAAAGAAACTTgcctctaggccctagggctctcaggca

aggaggcTAAGAAGGAATCCTTTGCTGTAGCCTTTTGGATTTAGGTTTCTCAGCTT

ATCTATCCCTCAGAGAAGTGTGTCTATGTCCCTTTTCTGTCCCTCTgcctcACCCC

ACCCCAACATTCCAACCTAGGGTAGGGGGAGGTCAgtatacacaaagccctctgtgtaaggg

gtggtatgtgtccccccaccccctacccagagtatacaatgcccctctgctccatgcccctgccaccctccc

caccacctctcaattgcacatgccaggctgcaattggtcactggctcaggacagccccctcatgctggggatcc

aggggattttaagcaggttccagaaaacaccactCAgttccttgtcccccgctctctccaccccacagacgct

ctgccaagcttgcatgcctgcaggtcgactctagaggatccagatc3'

+84

Figure 5. Model for Inhibition of P_0 Transcription by T Antigen and c-Jun

(A) The P_0 promoter region and various T antigen (Tag; I, II, III) and AP-1-binding elements are shown. Binding of T antigen to sites I and II and binding of c-Jun and T antigen to site III (Tag, AP-1 sites) are also indicated. Numbering is relative to the start site (+1).

(B) The DNA sequence of the myelin P_0 promoter (Lemke et al., 1988) with various T antigen sites (lowercase, underlined) and c-Jun-binding elements (bold, uppercase, underlined) are indicated. The T antigen-bound region (uppercase, bold) and the 24 bp spacer (lowercase, bold italics) are also indicated. The transcription start site is represented in bold uppercase italics.

protein p53 (Lane and Crawford, 1979; Linzer and Levine, 1979), transcription factor AP-2 (Mitchell et al., 1987), and the retinoblastoma susceptibility gene product p107 (DeCaprio et al., 1988; Moran, 1988). c-Jun dimerizes with other bZIP family members such as c-Fos (Rauscher et al., 1988), Fra-1 (Cohen et al., 1989), Fos-B (Zerial et al., 1989), Jun-B, and Jun-D (Nakabeppu et al., 1988), and with CREB protein (Benbrook and Jones 1990) via the leucine zipper (Landschulz et al., 1988) region. It is not known what regions within the T antigen and c-Jun mediate the interaction observed. One possibility is that c-Jun has a domain similar to that in p53 or Rb, or alternatively, c-Jun and large T antigen may interact via the leucine zipper region, since T antigen has a leucine-arganine-rich region between amino acids 345 and 370, which partially resembles a leucine zipper (Loeber et al., 1989). Interestingly, an antibody directed against p53 did not immunoprecipitate c-Jun (unpublished data), making it unlikely that T antigen, p53, and c-Jun exist in a ternary complex. Also in the immunoprecipitation studies, we were unable to coimmunoprecipitate large T antigen with c-Fos antibodies (Figure 1, lane 4).

Large T antigen has been shown to bind to at least two copies of the 5′-GAGGC-3′ pentanucleotide sequences (Tjian, 1978; Tegtmeyer et al., 1983) on the SV40 promoter. By using an immuno-DNA binding assay, we have shown that purified T antigen is able to bind to the P_0 promoter in the region from position −452 to −206 (Figures 2A and 2B). This region has two pentanucleotide sequences on the sense strand separated by a 24 bp spacer (−383 to −350; Figure 5B). However, one of the pentameric sequences is in the antisense orientation 5′-GCCTC-3′ (from position −354 to −350). Previous studies have indicated that orientation of the pentanucleotide sequence, as well as a spacer of 7 bp, necessitates high affinity binding to the SV40 early promoter (Ryder et al., 1985). It is likely that the arrangement of the pentanucleotide elements in the P_0 promoter reduces its affinity for T antigen. Such a reduced affinity may explain our inability to detect T antigen binding to the P_0 promoter by DNAase I footprinting (Figure 4, lane 3) and may also account for the inability of T antigen alone to alter significantly P_0 transcription.

The transcription factor c-Jun has been shown to bind to the consensus sequence TGAG/CTCA (Bohmann et al., 1987; Chiu et al., 1988; Angel et al., 1988); nevertheless, c-Jun is able to bind to DNA elements that differ from this reported consensus sequence

(Jones et al., 1988; Spandidos et al., 1989; Timmers et al., 1990). Although the 5′ flanking sequences do not contain any AP-1 consensus elements (Lemke et al., 1988), our results clearly indicate that c-Jun is capable of binding to the P_0 promoter independent of T antigen, as determined both by immuno-DNA binding (Figure 2B, lane 9) and by DNAase I footprinting (Figure 3, lane 4) assays. In the presence of T antigen, the ability of c-Jun to bind to the same region, 5′-GGGGTGAGGCAGAGG-3′ (from position −259 to −243) remained unaltered although enhanced protection was also observed from positions −241 to −230 (FP III) and −219 to −210 (FP IV). These findings indicate that the T antigen–c-Jun complex recognizes the c-Jun-binding elements on the P_0 promoter, although additional protection was found with the inclusion of T antigen. Interestingly, the c-Jun element contains within it one of the pentanucleotide sequence (site 3, from position −254 to −250). However, the role of this particular element remains unclear, since T antigen did not bind to this site as evidenced by immunobinding assays (Figure 2B, lanes 3 and 4).

In conclusion, our findings suggest that repression of the P_0 promoter is regulated by a T antigen–c-Jun complex that binds to the P_0 promoter and inhibits P_0 expression at a transcriptional level (Figure 5A). The fact that large T antigen and c-Jun form a complex and that this complex mediates repression of the P_0 gene raises a cautionary note for the study of gene regulation in mammalian cells when T antigen is used as an immortalizing oncoprotein, since T antigen may be affecting expression of the gene of interest. P_0 protein is a member of the immunoglobulin superfamily and plays a role in the compaction of myelin by homophilic interactions (Filbin et al., 1990; D'Urso et al., 1990). The lack of this protein has been shown to result in hypomyelination in the peripheral nervous system (Giese et al., 1992). Our results indicate that T antigen in concert with c-Jun represses P_0 expression and may well be the molecular mechanism underlying the observed demyelination and hypomyelination seen in the peripheral nervous system in transgenic mice expressing papovaviral large T antigen. Finally, with regard to the human demyelinating disease, progressive multifocal leukoencephalopathy, for which the primary etiological agent has been shown to be the polyomavirus JC viral T antigen protein, it is possible that a similar mechanism may operate for other myelin proteins in the CNS.

Experimental Procedures

Plasmids

pP_0cat reporter plasmid was obtained from G. Lemke, and pRSVc-jun was a gift from K. Ryder. pRSVtag was constructed by subcloning the HindIII–BamHI fragment of SV40 (strain 776, nucleotides 5171–2533) in place of the the HindIII–BamHI *cat* gene fragment in pRSVcat (Gorman et al., 1982). pOR.HKO plasmid containing the SV40 origin of replication (HindIII–KpnI) has been described previously (Li et al., 1986).

Cell Culture

Rat Schwann cell lines (Tennekoon et al., 1987; Peden et al., 1989) and secondary Schwann cells (Brockes et al., 1979) used for transfections were grown in 10 cm dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (DMEM-10). MT_4H1 and $MT_{wt}SV$ cell lines were maintained in the presence of 100 μM $ZnCl_2$.

DNA Transfection and Transient Cat Assays

Schwann cells were grown in DMEM-10 at 1×10^6 cells per 10 cm tissue culture dish for 24 hr before DNA transfection. Cells were transfected by the calcium phosphate precipitation technique (Gorman et al., 1982) and exposed to the precipitate for 8 hr. Cultures were then washed with phosphate-buffered saline and incubated with fresh medium. After 24 hr, cells were harvested, and Cat activity was determined as described by Gorman et al. (1982).

Cell Labeling and Immunoprecipitation

Confluent 10 cm dishes of either secondary or immortalized Schwann cells were labeled with 0.5 μCi/ml of trans ^{35}S label (ICN Radiochemicals) in a total of 5 ml of L-methionine- and L-cysteine-free Dulbecco-Vogt modified Eagle's medium. For 1 hr prior to labeling, cells were incubated in the same medium containing 5% dialyzed fetal bovine serum. Cells were labeled for 6–8 hr, and nuclei were isolated (Dignam et al., 1983) and lysed in 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 0.5% Nonidet P-40, 1% aprotinin (Sigma), and 0.3 mM phenylmethylsulfonyl fluoride (Sigma). Depending on the experiment, various amounts of anti-c-Jun polyclonal antibody (Oncogene Science), anti-T antigen monoclonal antibody PAb419 (Harlow et al., 1981), or normal rabbit antiserum were added to the nuclear lysates. When monoclonal antibodies were used, appropriate secondary antibodies were added before addition of protein A Sepharose. The antigen–antibody complexes were collected by addition of 5% protein A Sepharose (Pharmacia). Pellets obtained were washed 3 times with 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Precipitated proteins were analyzed by electrophoresis on 10% polyacrylamide SDS gels.

Western Blot Analysis

Cells were lysed, and nuclei were isolated as above. After immunoprecipitation, proteins were separated on a 10% SDS–polyacrylamide gel. The proteins were then transferred onto nitrocellulose. The filter was blocked in Blotto for 2 hr before the addition of primary antibody, and the ECL kit (Amersham) used to detect c-Jun or large T antigen.

DNA–Protein Binding Immunoassay

The method used is as described by McKay (1981). The pP_0cat plasmid was digested with AccI, XbaI, and BglII, and the pOR.HKO (Li et al., 1986) containing the SV40 origin of replication was digested with BstNI. After two phenol–chloroform extractions, 100 μCi of [α-^{32}P]dATP (NEN Dupont) was added to the mixture along with 1 U of Klenow DNA polymerase I (BRL) and incubated at room temperature for 15 min. The reaction was stopped by heat inactivation at 70°C for 5 min. Labeled fragments were separated from unincorporated nucleotides by two rounds of ethanol precipitation. One to two nanograms (10^5 cpm) of labeled fragments were incubated with 10–20 ng of purified large T antigen (gift from J. Pipas and A. Castelino) or recombinant c-Jun (Promega) for 60 min on ice. Following this incubation, 2 μl of large T antigen monoclonal antibody PAb419 or polyclonal c-Jun antibodies were added, and the mixture was incubated for an additional 30 min. Then, 3 μl of rabbit anti-mouse secondary antibody (Cappell Immunochemicals) was added after the addition of monoclonal anti-T antigen antibody. The immune complex was collected by adding 25 μl of a 50% slurry of protein A Sepharose (Pharmacia). The pellet was washed 3 times with 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, and 0.1% Noni-

det P-40. The bound DNA–protein complex was analyzed by electrophoresis on a 2% agarose gel, fixed in 10% acetic acid, dried, and exposed to Kodak XAR film. For further details, see McKay (1981) and Hinzpeter et al. (1986).

PCR-Generated Fragments

PCR cycling conditions used for amplification of each fragment were 96°C (1.5 min), 55°C (2 min), and 72°C (2.5 min), for 30 cycles. PCR-generated fragments 1–3 containing two or three T antigen binding sites (see Figure 5) were prepared by using 10 pmol of each oligonucleotide (see below) in a 50 μl reaction volume containing 100 ng of pP_0cat template, 3 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.3 at 20° C), 25 mM KCl, 200 μM dNTPs, and 2 U of Taq polymerase (Perkin Elmer). Amplified fragments were loaded on a 1.5% agarose gel and gel purified with GeneClean (Bio 101). Each fragment was then treated with 5 U of polynucleotide T4 kinase (NEB) and end labeled with [α-^{32}P]ATP (NEN Dupont). Oligonucleotides were synthesized by using the PCR mate oligosynthesizer (Applied Bio Science). Fragment 1 (−506 to −257) was synthesized by use of oligonucleotide sense GAATCTCCCAGGATG position −506 to −491 and antisense AGGGACAGAAAAGGG position −271 to −257; fragment 2 (−378 to −186) was synthesized with oligonucleotides sense TAGGCCCTAGGGCTC position −378 to −364 and antisense TTACACAGAGGGCTT position −201 to −186; and fragment 3 (−410 to −730) was synthesized with oligonucleotides sense GAATCTCCCAGGATG position −506 to −491 and antisense TTACACAGAGGGCTT position −201 to −186.

In Vitro Transcription Assay

In vitro transcription reactions were performed as outlined by Promega. Briefly, 8 U of HeLa cell extract (Promega), along with 500 ng of pP_0cat were incubated at 30°C for 1 hr in a reaction mixture containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 3 mM $MgCl_2$. An [α-^{32}P]ATP-labeled oligonucleotide primer (31 nucleotides) complementary to nucleotides 4930–4961 of the *cat* gene in pSV2cat (Gorman et al., 1982) was used to analyze the transcripts synthesized. Reverse transcription and primer extension were performed according to Jones et al. (1985). The extension products were analyzed on a denaturing polyacrylamide gel, and their lengths were measured relative to the migration of adjacent dideoxy sequencing products.

DNAase I Footprinting

Probes for DNAase I footprinting were generated by 5′ end labeling of oligonucleotide GAATCTCCCAGGATG (sense strand, from position −506 to −491) or oligonucleotide TTACACAGAGGGCTT (antisense strand, from position −201 to −186) with [α-^{32}P]ATP and polynucleotide T4 kinase. Subsequently, one end-labeled and one nonlabeled primer were used in a standard PCR reaction (see above) with 10 pmol of each primer and 500 ng of P_0cat DNA as a template. This generated a fragment from position −506 to −186 with either sense or antisense strand 5′ end labeled. PCR–generated fragments were gel purified before treatment with DNAase I. Binding reactions and DNAase I treatment were followed by using the Promega footprinting kit with 10–30 ng of purified c-Jun protein (Promega) and 0.5–1.5 μg of purified T antigen (gift from J. Pipas and A. Castelino). Sequencing ladder reactions were performed as outlined by Maxam and Gilbert (1980).

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