Activity of Simian DNA-Binding Factors Is Altered in the Presence of Simian Virus 40 (SV40) Early Proteins: Characterization of Factors Binding to Elements Involved in Activation of the SV40 Late Promoter

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The early proteins of simian virus 40 (SV40) large T and small t antigen (T/t antigen) can each cause the transcriptional activation of a variety of cellular and viral promoters. We showed previously that simian cellular DNA-binding factors (the Band A factors) bind to sequences within the SV40 late promoter which are important for transcriptional activation in the presence of the SV40 early proteins. Band A factors isolated from simian cells which produce T/t antigen (COS cells or SV40-infected CV-1 cells) have altered binding properties in comparison with the factors from normal simian cells (CV-1). This suggests that the transcriptional activation mediated by T/t antigen may be due to either modification of existing factors or induction of new members of a family of factors. We have purified the Band A factors from both COS and CV-1 cells and have determined the binding site by methylation interference and DNase protection footprinting. The COS cell factors have altered chromatographic properties on ion-exchange columns and have higher-molecular-weight forms than the CV-1 cell factors. Major forms of the CV-1 factors migrate between 20 and 24 kilodaltons, while the COS factors migrate between 20 and 28 kilodaltons. The binding sites for the factors from CV-1 and COS cells are similar, covering a rather broad region within the 72-base-pair repeat comprising the AP-1 site and the two-octamer binding protein (OBP100/Oct 1) sites, OBP I and OBP II. Specific binding competition analyses indicate that the two general regions within the binding site (the AP-1-OBP II site and the OBP I site) each retain partial binding ability; however, the factors bind best when the two regions are adjacent in a relatively specific spatial arrangement. The binding site for the Band A factors corresponds very well to sequences necessary for the activation of the late promoter as defined by deletion and base substitution mutagenesis studies (J. M. Keller and J. C. Alwine, *Mol. Cell. Biol.* 5:1859-1869, 1985; E. May, F. Omilli, M. Emoult-Lange, M. Zenke, and P. Chambon, *Nucleic Acids Res.* 15:2445-2461, 1987). These data, in combination with the data showing that the Band A factors are modified or induced in the presence of T/t antigen, strongly suggest that T/t antigen mediates its transcriptional activation function, at least in part, through the Band A factors.

The double-stranded DNA viruses which replicate in the nucleus of eucaryotic cells (the herpesviruses, adenoviruses, and papovaviruses) have played a pivotal role in the definition of eucaryotic promoter and enhancer structure, as well as in the mechanisms of transcriptional control. Although these viruses differ greatly in size, host range, and genetic complexity, they display one feature in common: the temporal nature of their gene expression control. Each virus expresses its genes in a specific order, for example, early genes and late genes. To preserve the sequential expression of the genes, each of these viruses initially expresses (or presents as part of the virion) viral proteins which mediate the activation of subsequent viral gene expression. This general scheme insures temporal order of gene expression. Examples of such viral *trans*-acting proteins include the simian virus 40 (SV40) large T antigen (10, 33) and small t antigen (38), the E2 proteins of bovine papillomavirus and human papillomavirus (25, 28), adenovirus E1a (30, 31, 41), pseudorabies virus IE protein (29, 30), and herpes simplex virus ICP4 and VP16 (12, 15, 18, 35, 36).

It appears that the viral *trans*-acting proteins mediate transcriptional activation through the induction or activation of cellular transcription factors. This has been demonstrated for adenovirus E1a (37, 43, 49), herpesvirus VP16 (36), and pseudorabies IE protein (1, 2) and bovine papillomavirus E2 (25, 28). SV40 early proteins (T/t antigen) appear to use a similar strategy. We have previously shown (20) that T/t antigen mediates the modification or induction of at least one family of simian DNA-binding factors, the Band A factors. These factors bind to a region of the SV40 late promoter which is necessary for the activation of the late promoter in the presence of T/t antigen (17, 34, 40). We previously found that the Band A factors in nuclear extracts from simian cells which produce T/t antigen (COS or infected CV-1 cells) showed increased stability of binding to their recognition sites compared with the Band A factors from normal simian cells (CV-1 cells). In addition, the factors from COS or infected CV-1 cells demonstrated a microheterogeneous banding pattern when examined by DNA fragment retention analyses. Together, these results suggest either modification of the Band A factors or induction of new members of a family of Band A factors by T/t antigen.

In the present communication we report the purification and characterization of the Band A factors from both CV-1

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FIG. 1. The promoter region of SV40 and the binding pattern detected in DNA fragment retention analyses by using the +Tau probe with COS and CV-1 nuclear extracts. The promoter region shows the detailed structures of the early and late promoters, which are discussed in the text. Briefly, many laboratories have defined elements involved in the transcription of late RNA (5, 7, 8, 11, 16, 17, 20, 22, 24, 26, 27, 34, 40, 44, 46). These include an intragenic region (7, 26, 44) which has been proposed to function in attenuating late RNA transcription (26, 44). The regions defined by our laboratory (4, 5, 20, 34) have been given Greek-letter names; of these, the tau and delta regions are necessary for the activation of the late promoter in the presence of T/t antigen (34). These elements overlap Domains I and II, similarly defined by Ernoul-Lange et al. (17). The tau and delta regions are contained in the +Tau probe used in DNA fragment retention analyses. An example of the results of such analyses in which COS and CV-1 nuclear extracts were used is shown on the right. There are three Band A factor-binding sites; they are denoted TABS. The TABS regions include the AP-1, OBP I, and OBP II sites, as defined in Band A factor footprinting data presented herein. There are two identical TABS in the 72-bp repeats and a degenerate one in the Ori region. At the bottom of the figure, the +Tau probe is partially expanded to show that it contains many overlapping binding sites for known transcription factors.

and COS cells. The Band A factor is relatively small in both types of cells, and is more positively charged in T/t antigen-containing cells. Footprinting of the factor indicates a broad binding site covering the adjacent AP-1 and OBP100/Oct 1 (45) sites within the late promoter. Binding competition studies indicate that both of these sites must be adjacent in a specific spatial orientation for optimal binding. These data together with our previous experiments strongly suggest that the Band A factors are cellular factors through which T/t antigen may mediate transcriptional activation.

MATERIALS AND METHODS

Cells and plasmids. Experiments were performed with the established African green monkey kidney cell line, CV-1, and a derivative of this line, COS cells, which are transformed by an origin-defective SV40 and constitutively express wild-type T/t antigen. CV-1 cells were grown in Dulbecco modified essential medium (GIBCO) supplemented with 10% (vol/vol) fetal calf serum. After eight passages, cells were discarded and new cells were revived from liquid nitrogen. COS cells were similarly maintained, except that Dulbecco modified essential medium was supplemented with 4.5 g of glucose per liter and 7.5 to 10% fetal calf serum.

All plasmids were maintained in *Escherichia coli* strain HB101 by using LB broth or M9 medium and ampicillin

selection. Plasmids were prepared as previously described (39).

Probes and competitors for DNA fragment retention analysis. DNA fragments used either as probes or specific competitors were generated from recombinant plasmids as previously described (20). The specific DNA fragment used as a probe in these experiments was the +Tau probe shown in Fig. 1. For the preparation of all probe or competitor fragments, we filled in or digested sticky ends so that they would be blunt. The +Tau probe was made by linearizing pL540-Cat (34) at the inserted *Xho*I site (at SV40 nucleotide 154) and end labeling with [α - 32 P]dCTP by filling in the recessed 3' end. Alternatively, the fragments were 5' end labeled by using [γ - 32 P]ATP. DNA which was end labeled by either procedure was then digested with *Kpn*I (SV40 nucleotide 294), producing a 144-bp fragment which was purified by electrophoresis on a 10% polyacrylamide gel; digestion was followed by binding and elution from DE52 membrane (Schleicher & Schuell). Other competitor fragments, described below, were analogous to the +Tau probe and were isolated in a similar manner.

Nuclear extracts. Nuclear extracts used in DNA-binding assays were prepared from CV-1P and COS cells by the method described by Dignam et al. (14), with the modifications of Wildeman et al. (48). All steps for extract production were performed at 4°C. Cells just confluent were washed two times with phosphate-buffered saline (GIBCO), scraped into

phosphate-buffered saline, and centrifuged at 2,000 rpm. Consolidated cells were suspended for 10 min in a volume of buffer A (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid], pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol) equivalent to five times the packed-cell volume. Cells were centrifuged at 2,000 rpm (SS-34 rotor, Sorvall) and the cell pellet was resuspended in a volume of fresh buffer A equivalent to two times the cell pellet volume. The cells were then lysed by Dounce homogenization by using a B pestle; cell lysis was monitored microscopically. Nuclei were removed from cell debris and the cytoplasm by centrifugation at 14,500 rpm (SS-34 rotor, Sorvall) for 20 min. The pelleted nuclei were suspended in 3 ml of buffer B (20 mM HEPES, pH 7.9, 25% glycerol [vol/vol], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol) per 10^9 cells by 50 strokes with a B pestle in a Dounce homogenizer and gentle stirring for 30 min to lyse the nuclear membranes. Particulate matter was removed by centrifugation at 14,500 rpm for 30 min. The supernatant was collected and precipitated by the slow addition of 0.33 g of ammonium sulfate per ml with stirring. The precipitant was pelleted at 14,500 rpm, and the supernatant was discarded. The pellet was suspended in 1/12th the original volume of buffer C (20 mM HEPES, pH 7.9, 17% glycerol [vol/vol], 20 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol) and dialyzed overnight against 100 volumes of buffer C, changed twice. Dialysis was performed in nitrocellulose ultrathimbles (Schleicher & Schuell). Final protein concentrations were determined by using the Bio-Rad protein assay (9). Extracts were quick frozen in liquid nitrogen and stored at $-80^\circ C$ until needed, at which time extracts were thawed at $4^\circ C$ and diluted as necessary with buffer C.

Purification of the Band A factor. CV-1 and COS nuclear extracts were partially purified with the cation-exchange resin S-Sepharose (Pharmacia). Large plastic pipette tips (P1000, Rainin) were siliconized, and the tip was plugged with siliconized glass wool. S-Sepharose exchange resin (500- to 700- μ l packed volume) was added to the plugged tip and equilibrated in buffer C. Nuclear extract (2 mg) was diluted in 500 μ l of buffer C and loaded onto the column. Eluate was collected and passed over the column two more times prior to the addition of 1 ml of buffer C, which was collected with the input volume. This fraction was designated S-Sepharose flow-through. Step gradient elution was performed by passing 1.5-ml volumes of buffer C containing increasing concentrations of KCl. Fractions were concentrated by centrifugation in Centricon-30 concentrators (Amicon Corp.) and reequilibrated in binding buffer (12 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA, 0.6 mM dithiothreitol, 12% glycerol) by successive dilution and concentration. Final fractions were analyzed by DNA fragment retention analysis (see below).

The active S-Sepharose fractions were pooled and further purified by specific DNA affinity chromatography (33). The specific complementary oligonucleotides used corresponded to SV40 nucleotides 182 to 201. The DNA affinity resin was packed into a siliconized pipette tip, as described above. The column was equilibrated in binding buffer. The S-Sepharose fractions were diluted to a final volume of 500 μ l of binding buffer supplemented with 10 μ g of poly(dI-dC):poly(dI-dC) (Pharmacia) before loading onto the affinity column. The column was kept closed while the resin was dispersed by pipetting and then allowed to settle for 30 min before allowing the column to flow. The input volume was collected along with an additional 1-ml wash of binding buffer. The column was again closed, and the resin was dispersed with

500 μ l of 1 M KCl in binding buffer and allowed to settle for 30 min. The high-salt elution fraction was collected, and the column was eluted with an additional 1 ml of 1M KCl in binding buffer; the combined high-salt fractions were designated the affinity-purified fraction (APF). The APF was concentrated and reequilibrated, as described above, in binding buffer and analyzed by DNA fragment retention analysis.

DNA fragment retention analysis. The factor binding reactions and subsequent analyses were performed as previously described (20). For binding competition experiments, all components of the reaction, including 0.5 to 10 ng of specific competitor, were mixed together prior to the addition of nuclear extract. After a 20-min binding incubation at $4^\circ C$, the reaction mixes were loaded directly onto 17-cm, 1.2-mm thick, 4% polyacrylamide gels (acrylamide-bisacrylamide [30:1]) which had been prerun at 25 mA for 1 to 2 h at $4^\circ C$ in a recirculated running buffer of 13.4 mM Tris (pH 7.9)–6.6 mM sodium acetate–2 mM EDTA. Electrophoresis was performed at 35 mA at $4^\circ C$ for 3 to 4 h. Gels were dried and visualized by autoradiography.

Samples requiring high-resolution analysis (20) were prepared as described above, except that the nonspecific competitor was changed to poly(dI):poly(dC), which allowed better resolution than poly(dI-dC):poly(dI-dC) on longer gels. After incubation, the samples were loaded onto a 40-cm, 1.3-mm thick, 5.5% polyacrylamide gel (acrylamide-bisacrylamide [30:0.5]) which had been prerun with recirculating buffer as above. Electrophoresis was performed at 40 mA at $4^\circ C$ for 6 to 7 h.

DNase and methylation interference footprint analyses. DNase and methylation interference footprint analyses (19, 21) were performed by using the +Tau probe (6,000 cpm) singly labeled either on the early strand (3' end labeled) or the late strand (5' end labeled). Thus the label was always on the same end of the probe fragment, making the orientation of both strands the same in denaturing gel electrophoresis.

Binding reactions for DNase footprinting were performed as described above. For digestion, 1 μ l of DNase (0.025 μ g/ μ l in ice-cold 20 mM sodium acetate, pH 7.4, 5 mM $CaCl_2$) was added to each reaction and allowed to incubate for 0.5 min at $4^\circ C$. Digestion was stopped by adding 21 μ l of Stop Buffer (1% sodium dodecyl sulfate [SDS], 20 mM EDTA, 40 μ g of tRNA per ml). After phenol extraction and ethanol precipitation, the samples were analyzed on a sequencing gel.

Methylation interference assays were modifications of those described by Gilman et al. (21), with 60,000 cpm of methylated +Tau probe, labeled as described above. Probes were used in DNA fragment retardation assays with either crude extracts or APFs. Protein concentrations used were approximately 10 times the normal amount. After DNA fragment retention electrophoresis, the wet gels were autoradiographed to visualize the free and bound probe (Band A). Bands representing free and bound probe were excised, and the DNA was eluted. The isolated DNA was then cleaved at methylated A and G residues. Recovered samples were counted, and equal counts of free and bound fractions were analyzed on sequencing gels. The resulting autoradiograms were analyzed by laser densitometry; by computer analysis, the scans were normalized to bands which were not involved in binding, thus allowing relative comparisons.

SDS-polyacrylamide gel electrophoresis (PAGE) separation and renaturation of the Band A factor. Renaturation of DNA-binding activity from SDS-polyacrylamide gels was performed as previously described (23). Briefly, a 300- μ g

FIG. 2. Purification of the Band A factors. COS (A) and CV-1 (B) nuclear extracts were fractionated by S-Sepharose ion-exchange chromatography. Fractions were eluted by a KCl step gradient and were analyzed by DNA fragment retention analyses by using the +Tau probe. The lane numbers indicate the concentration of KCl (mM) used to elute the fraction: IN (input) denotes the nuclear extract used for the fractionation; S-FT is the flowthrough fraction. COS and CV-1 S-Sepharose fractions which showed Band A factor activity were analyzed by high-resolution DNA fragment retention analysis (C) (see Materials and Methods). The active S-Sepharose fractions from COS and CV-1 extracts were then separately pooled and fractionated by specific DNA affinity chromatography. The oligonucleotide used for the fractionation is shown in Fig. 5 (AP-1 oligo). High-resolution DNA fragment retention analysis of the pooled S-Sepharose fractions (IN, input) and the fraction retained on the AP-1 oligo column and eluted in high salt (APF) (D) is shown.

sample of COS or CV-1 nuclear extract was denatured in SDS sample buffer and electrophoretically fractionated on a preparative 10% SDS-polyacrylamide gel (4% stacking gel). The gel was run for 15 h at 10 mA. The lanes were sliced into 0.5-cm segments. Each segment was crushed with a spatula in 1.7-ml Eppendorf tubes; then, 1.2 ml of elution buffer (50 mM Tris hydrochloride, pH 7.9, 150 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1% SDS) was added. The samples were incubated with shaking at room temperature for 2 to 4 h. Gel fragments were sedimented by centrifugation, and 1 ml of supernatant was transferred to a 15-ml Corex tube. Protein was precipitated by addition of 4 volumes of cold acetone and storage at -20°C . Precipitated protein was sedimented by centrifugation at 10,000 rpm for 15 min in a Sorvall SS-34 rotor. The pellets were washed with 1 ml of 80% acetone to remove residual SDS, then air dried. The pellets were suspended in 100 μl of 6 M guanidinium HCl and incubated at room temperature for 30 min. The samples were renatured by overnight dialysis in binding buffer.

RESULTS

The late promoter and the Band A binding activity. Figure 1 shows the region of SV40 DNA between nucleotides 5143 and 650, which contains the origin of replication (Ori) (the last and first nucleotides of the circular genome, 5243 and 1, are within the Ori), the overlapping early and late promoters, and part of the late-coding region. Elements of the early promoter are the primary transcriptional start sites detected

early (E_e) and late (E_l) in infection, the TATA region, (TA), the 21-base-pair (bp) repeats, and the 72-bp repeat enhancers. In addition, Fig. 1 indicates the T-antigen-binding sites (I and II); these are the sites to which large T antigen binds in order to autoregulate early transcription and to initiate each round of DNA replication (6, 13, 42).

Work from our laboratory, as well as that of many others, has defined elements throughout the promoter region, as well as within the late-coding region, which play a role in the activity of the late promoter (4, 5, 7, 8, 11, 16, 17, 20, 22, 24, 26, 27, 34, 40, 44, 46). The regions defined in our previous studies (4, 5, 20, 34) are given Greek-letter names in Fig. 1. The most significant of these for the present studies are tau and delta, which play a significant role in late promoter activation in the presence of T antigen. Note the position of these elements within the 72-bp enhancer region. In our previous studies of factor binding within the tau-delta region (20), we used the +tau probe (Fig. 1) in DNA fragment retention analyses with nuclear extracts of CV-1 or COS cells. The routine results of such experiments are similar to those shown in Fig. 1. Binding competition studies showed that Band A is due to specific factor binding in the 5' one-third of the probe (the tau region) and Band B results from specific factor binding in the delta region (20). Our previous studies of the Band A factors indicate that they are activated or induced in the presence of T/t antigen. In this paper we deal primarily with the Band A factors; however, some evidence suggests that Band B may also be influenced by the presence of T/t antigen. The regions marked TABS

FIG. 3. Band A factor methylation interference footprinting in which COS and CV-1 nuclear extracts were used. The procedure for preparing the footprint autoradiograms and their analysis by laser densitometry is described in Materials and Methods and in Results. The dark lines indicate where the intensities of bands in the bound fraction were notably diminished compared with the free fraction. A compilation of the results is shown in Fig. 5.

(T/t activatable binding site) in Fig. 1 are the Band A factor-binding regions as defined in the present work.

S-Sepharose fractionation and DNA affinity purification of COS and CV-1 nuclear extracts. Nuclear extracts of COS and CV-1 cells were fractionated on the cationic-exchange resin S-Sepharose. Figure 2A and B show the DNA fragment retention analyses of the COS and CV-1 fractions, respectively, generated by a KCl step gradient; the number denoting each lane indicates the concentration of KCl used to elute that fraction. The majority of the Band A factor from COS cells eluted between 175 and 225 mM KCl (lanes 175, 200, and 225), whereas the Band A factor from CV-1 cells did not bind to the column and remained in the flowthrough (lane marked S-FT). These differences in chromatographic properties indicate that the COS cell factors, compared with the analogous factors from CV-1 cells, have either a greater positive charge or a conformational change which has exposed a positive surface. This supports our previous finding that there is a distinct difference in the properties of the Band A factor(s) between normal simian cells and those which contain T/t antigen.

In previous experiments (20) we used high-resolution DNA fragment retention analyses to demonstrate that the presence of T/t antigen in cells (either COS or SV40-infected CV-1 cells) resulted in a microheterogeneity of bands produced by binding of Band A factors, compared with a relatively simple banding pattern in uninfected CV-1 cells. Figure 2C shows the high-resolution DNA fragment reten-

tion analysis of the COS and CV-1 S-Sepharose fractions containing the Band A-binding activity. This analysis confirms with more highly purified proteins the differences previously reported between the binding activities from COS and CV-1 nuclear extracts. Specifically, the banding pattern observed with the purified COS Band A factor (175 to 225 mM KCl fractions) was microheterogeneous (3 to 4 bands), whereas the active CV-1 fraction (S-Sepharose flowthrough) produced a single band.

Possible effects of the presence of T/t antigen on the nature of the Band B factor(s) can also be seen by comparing the data in Fig. 2A and B. The COS Band B factor elutes predominantly in the 175-mM KCl fraction, whereas the CV-1 activity partitions in fractions 175 to 225. This difference is substantiated in the high-resolution gels, where there is a distinct migration difference noted between the Band B factors from COS and CV-1 cells.

The fractions containing the Band A activities from COS and CV-1 cells were further purified by sequence-specific DNA affinity chromatography (32). There are two different areas within the SV40 promoter region which contain Band A factor-binding sites (20). These areas are the 5' one-third of the +Tau probe (SV40 nucleotides 150 to 220) and a region near the SV40 Ori (SV40 nucleotides 1 to 41). A striking site of homology between these regions is the sequence TXGCTGACTAATT (nucleotides 182 to 194 and 39 to 27) containing an AP-1-binding site. For our initial purifications we used an oligonucleotide containing these

sequences (see AP-1 oligo) to generate a specific DNA affinity column. The results of specific DNA affinity purification of the S-Sepharose fractions containing the Band A factors are shown in Fig. 2D, which is a high resolution DNA fragment retention analysis of the input fraction (IN) and the APF which eluted from the DNA column in high salt. The COS and CV-1 APFs contained most of the Band A species present in S-Sepharose fractions; much of the microheterogeneity of COS remains through the purification. The CV-1 APF shows a second binding activity higher in the gel; this activity is not repeatably observed and has not been further studied. It should be noted that the APF lanes in Fig. 2D were derived from binding reactions done in the absence of nonspecific DNA competitor (poly[dI-dC]:poly[dI-dC]). This indicates that most nonspecific binding proteins have been removed; in more crude fractions the nonspecific competitor is needed for the probe to enter the gel and form specific bands. Silver-stained SDS-PAGE gels of the COS and CV-1 APFs indicated only five to six discrete bands of varied molecular weights (not shown). Analysis of the size of the Band A factors by SDS-PAGE is described below.

Band A factor footprint analyses. Our previous data suggested that the Band A factor may be binding near the AP-1 site in the tau region (Fig. 1). Methylation interference footprinting showed that the binding region was larger than expected. The methylated +Tau probe, labeled on the 5' or 3' ends, was used in a DNA fragment retention assay with nuclear extracts or the APFs from COS and CV-1 cells. After electrophoresis, the bands were visualized by wet-gel autoradiography and the bound (Band A) and unbound (free probe) bands were excised and eluted. The two populations of DNA were acid treated to cleave at methylated A and G residues. Equal numbers of counts of the final digests were separated on a sequencing gel. The crude nuclear extracts reproducibly yielded readable but somewhat weak footprints. In order to accurately establish the footprint and to correct for variability in counts loaded, the data were analyzed by laser densitometry (see Materials and Methods). The results of these comparisons are shown in Fig. 3 for crude nuclear extracts and in Fig. 4 for the COS APF. In each case, the thin lines indicate the scan of the free (unbound) fractions; the darker lines indicate where the intensity of bands in the bound fraction was significantly diminished in comparison with the free fraction. Interference was observed over a broad region between SV40 nucleotides 180 and 220 for CV-1 nuclear extracts (Fig. 3, left panel), between 186 and 223 for COS nuclear extracts (Fig. 3, right panel), and between 187 and 213 for the COS APF (Fig. 4). The methylation interference footprinting with the CV-1 APF (not shown) was not significantly different from that obtained with the crude nuclear extract.

The summary of the methylation interference footprinting data is shown in Fig. 5; the solid diamonds indicate the more strongly interfering nucleotides. Several points are indicated by the summary. (i) In both CV-1 and COS cells there is a dramatic effect of the A residues on the 3'-end-labeled probe, indicating that significant interactions occur between the proteins and DNA in the minor groove of the helix. (ii) Many of the same bases are involved in binding of factors from each cell type; however, there are clear differences. More bases on the late strand (the upper strand in Fig. 5) appear to be involved in COS factor binding. (iii) Our previous data comparing two different Band A factor-binding sites (discussed above) suggested that the AP-1 region may be the binding site for the Band A activity. Interfering bases are found in this region; however, strongly interfering se-

FIG. 4. Methylation interference footprinting in which affinity purified COS Band A factors were used. The data were gathered as described in the text and in the legend to Fig. 2. The thin lines indicate those regions where the relative band intensities were the same between the free and bound fractions. The dark lines indicate where the relative intensity of bands in the bound fraction were significantly diminished compared with the free fraction. A compilation of the results is shown in Fig. 3.

quences also correspond quite closely to the OBP100/Oct 1 binding sites (45; the OBP motifs, OBP I and OBP II). The AP-1/OBP II region is apparently a sufficient minimal binding site for the Band A factors, since the AP-1 oligonucleotide (Fig. 5) was efficient in specific DNA affinity chromatography of the factors (Fig. 2). This point is further examined below.

DNase footprinting of Band A factor binding with nuclear extracts and APFs from CV-1 cells was unsuccessful. This is likely due to a relatively rapid dissociation rate of the factors (20) which would allow DNase access to all regions of the probes. The dissociation rate of the CV-1 factors is very fast (20); however, the more stable binding of the COS factors, coupled with factor concentration, due to purification, allowed the use of amounts of affinity purified factors capable of driving the reaction toward binding. The results of DNase protection footprinting with the COS APF are shown in Fig. 6A; the densitometric analysis of the data was also performed and is shown in Fig. 6B. As a positive control for the footprinting reaction, we used a partially purified HeLa AP-1 fraction (a gift of D. Bohmann and R. Tjian). For the COS APF, protection and hypersensitive sites were noted between nucleotides 200 and 237 on the 5'-end-labeled strand and between nucleotides 186 and 235 on the 3'-end-labeled strand. The summary of the data is shown in Fig. 5 (affinity-

FIG. 5. Compilation of the footprinting data for the Band A factors. The sequence between SV40 nucleotides 151 and 250 within the +Tau probe is shown. The diamonds indicate A and G residues which, when methylated, interfere with Band A factor binding. The open diamonds indicate residues in which the effect of methylation was less pronounced, as judged from the data in Fig. 3. The bottom sequence shows both the methylation interference and DNase footprinting data in which the specific DNA affinity-purified Band A factors from COS cells were used (Fig. 4 and 6). The horizontal lines indicate regions protected from DNase attack; the vertical arrows indicate hypersensitive sites. At the bottom of the figure, the positions of the binding sites for AP-1 and OBP100/Oct 1 (OBP I and OBP II) are shown. In addition, the sequence contained in the AP-1 oligonucleotide used for specific DNA affinity chromatography (see Fig. 2) is indicated.

purified COS); horizontal arrows indicate protected regions, vertical arrows indicate hypersensitive sites. Overall, the DNase footprint data and methylation interference data with the COS APF suggest that the functional binding site for the Band A factors is a rather broad region including the adjacent AP-1 site and sites I and II for OBP100.

Sizing and properties of the Band A factor(s) with SDS-PAGE separation. Crude nuclear extracts of COS and CV-1 were fractionated on preparative SDS-polyacrylamide gels. The gel lanes were sliced into 0.5-cm sections, and the proteins were eluted and renatured (see Materials and Methods). The binding activities in the fractions were assayed by DNA fragment retention analysis by using the +Tau probe; the results are shown in Fig. 7. Clearly the Band A factors from both CV-1 and COS cells are heterogeneous low-molecular-weight proteins. The results from several experiments indicate that the molecular weight range of the CV-1 factors is 20,000 to 24,000, while the range for COS is between 20,000 to 28,000 or higher. In agreement with previous results, the presence of higher-molecular-weight species indicates altered forms of the factors in COS cells.

Other information can be gathered from the renatured SDS-PAGE gel fractions. The Band B activity, although faint in these samples, can be detected migrating, with a molecular weight of approximately 40,000. Furthermore, the fractionation of factors allowed the detection of several additional binding activities (bands c through f) in COS extracts which were apparently masked in the crude extracts. These activities have not been characterized; preliminary binding competition studies (not shown) indicate that

bands d and e may be specific binding activities, while the band f doublet is nonspecific.

Binding competition analysis of the Band A factor recognition region. The small size of the Band A factor compared with the relatively large binding site suggests that the Band A factors may function as multimers, recognizing more than one site. In order to gain insight into the binding mechanism, we performed binding competition experiments to determine whether the two definable regions within the binding site (the OBP II/AP-1 region and OBP I site) each had binding activity and whether they may function cooperatively.

A series of competition fragments based on the wild-type +Tau fragment were generated as shown in Fig. 8. In one set of competitors, an *Sph*I site between the OBP I site and the OBP II/AP-1 region was used to individually remove each region and replace it with nonspecific plasmid DNA. The -TauX competitor removes the OBP II/AP-1 region but leaves the OBP I site intact, while the inverse is the case for the +TauX competitor. In a second set of competitors, the use of the *Sph*I site allowed the OBP II/AP-1 and OBP I regions to be moved 4 base pairs closer together (-4; by blunting the *Sph*I site) or further apart (+4, +6, +12; by filling in the *Sph*I site or by the insertion of different-sized linker or spacer sequences). These manipulations left the OBP I and OBP II sites largely intact, as indicated by the underlines in Fig. 8; however, as noted by x marks, some of the bases at the 3' end of OBP II and the 5' end of OBP I were altered in some constructions. It has been suggested that such alterations affect the binding of HeLa OBP100/Oct 1 (45).

FIG. 6. (A) DNase footprint analysis with the affinity-purified COS Band A factors. The left panel shows the footprint data using the COS APF (lanes 1 to 4) and a partially purified HeLa AP-1 fraction (a gift of D. Bohmann and R. Tjian), which was included as a positive control (lanes 5 to 7). Lanes M1 and M2, Specific markers. Lane 1, Free 5'-end-labeled probe; lane 2, bound 5'-end-labeled probe; lane 3, free 3'-end-labeled probe; lane 4, bound 3'-end-labeled probe. Regions of protection (brackets) and hypersensitivity (arrows) are indicated. (B) Densitometry analysis of the data. The darker lines indicate where the relative intensity of bands in the bound fraction were significantly different, either greater or less, compared with the free probe. The horizontal lines indicate the first and last nucleotide in the footprint region. A compilation of the results is shown in Fig. 5.

Increasing amounts of each competitor were added to a constant amount of [32 P]+Tau probe and analyzed by DNA fragment retention analysis by using COS nuclear extract and, for a few competitors, the COS APF. The intensity of the retained probe band was measured by densitometry and a competition curve (negative log percentage bound versus femtomole of competitor) was generated. A self competition curve was also generated by using unlabeled wild-type +Tau fragment as competitor; the slope of this curve (negative log percentage bound per femtomole of competitor) was calculated and designated to have a competitive efficiency of 1.00, i.e., 100% efficiency of competition. Slopes were calculated for all the competitors and compared with the slope of the +Tau competition curve in order to calculate relative competitive efficiencies.

The results (Fig. 8) indicate that the individual OBP II/AP-1 region and the OBP I site (in +TauX and -TauX, respectively) retained some competitive ability (24 and 45% of the wild-type level, respectively), but neither competes as well as when the two regions are together, as in the wild-type condition. This finding was confirmed by the results with the competitors which moved the regions closer and farther apart. Moving the regions 4 base pairs closer (-4) lowered the competitive efficiency moderately when tested with crude nuclear extract, but this had little effect on competitive efficiency with the APF. Likewise, little change in competitive efficiency occurred when the regions were moved 4 base pairs (+4) apart. However, moving the regions 6 or 12

base pairs apart (+6 and +12) caused a considerable drop in competitive efficiency.

These data suggest that the two general regions of the Band A factor-binding site have individual binding ability; however, binding is most efficient when the two regions are adjacent in a relatively specific spatial arrangement.

DISCUSSION

Figure 9 shows a diagrammatic representation of the Tau-Delta region, including the domains I and II, previously defined genetically as being required for late promoter activation in the presence of T/t antigen (17, 34, 40). The sites for the AP-1 and OBP100/Oct I (OBP I and OBP II) are indicated. May et al. (40) have defined the activity of the late promoter in the presence of T antigen by using base substitution mutants through this region. As indicated in Fig. 9, they found that mutations within OBP II/AP-1 region resulted in 20 to 60% decreases in late promoter activity, while mutations within the OBP I region resulted in a uniform 80% decrease in late promoter activity. The regions defined by these data agree very well with our DNase and methylation interference footprinting data in which the affinity-purified COS Band A factor was used. In addition, the base substitution mutation results correspond well with our binding competition data, which indicated that the OBP I region was a better competitor than the OBP II/AP-1 region. This precise agreement between sequences defined by two dif-

FIG. 7. DNA fragment retention analysis of CV-1 and COS nuclear extracts fractionated on SDS-polyacrylamide gels. CV-1 and COS nuclear extracts were electrophoretically separated on SDS-polyacrylamide gels. The gels were cut into 0.5-cm fractions, and the proteins were eluted from each fraction and renatured as described in Materials and Methods. The renatured proteins were tested for binding activity by DNA fragment retention analysis by using the +Tau probe. The numbers over the lanes indicate the position, in centimeters, of the gel slice from which the proteins were eluted. Across the top of each panel, the migration positions of marker proteins are indicated by molecular weight. In addition to Bands A and B, several new bands were detected after fractionation of the COS extract; they are marked bands c to f.

ferent methods strongly suggests that the Band A factors play a significant role in the activation of the late promoter. In addition, we have shown in this and previous studies (20) that the Band A factors are modified or induced by the presence of T/t antigen. Specifically, we find that in the presence of T/t antigen, the Band A factors: (i) bind more stably to their target sites (20; in the present study the binding instability of the CV-1 Band A factor was clearly indicated by the inability to generate a DNase footprint); (ii) have higher-molecular-weight forms and cause microheterogeneous banding in DNA fragment retention analyses (this is indicative of modifications or newly induced members of a family of factors); and (iii) have altered chromatographic properties on ion-exchange columns, again indicating modifications or newly induced forms.

Overall, the data show that a promoter region necessary for late promoter activation in the presence of T/t antigen is the binding site for factors which are altered in the presence

of T/t antigen. This concurrence of data strongly suggest that the Band A factors are targeted cellular transcription factors through which T/t antigen functions for the activation of the late promoter and possibly cellular promoters as well.

The binding site for the Band A factors is quite similar to the site(s) defined for the HeLa cell factor OPB100/Oct 1 (45). The site includes the partially overlapping AP-1 and OBPII sites and the adjacent OBPI site. In Fig. 1 this combined site is called TABS. There are two identical TABS in each 72-bp repeat and one with partial sequence homology in the region of the Ori. The Ori TABS is oriented opposite to the TABS in the 72-bp repeats. The Band A factors require the AP-1/OBPII and OBPI regions to be adjacent in a relatively exact spatial orientation for the most efficient binding. Binding may be cooperative.

The existence of three TABS suggests that the alterations of the Band A factors may mediate effects on late transcription from several locations in the promoter. Although dele-

		RELATIVE COMPETITIVE EFFICIENCY*	
		Nuc. Ext.	APF
CTTTGCATACTTCTGCCT-- -Tau		0	0
-----GACTCACTATAGGGAGACCGGAAGCTTGCATGCTTTGCATACTTCTGCCT-- -TauX		0.45	NT
-----TGTTGCTGACTAATTGAGATGCATGCAAGCTTCCGGTCTCCCT-- +TauX		0.24	NT
<div style="text-align: center;"> </div>			
-----TGTTGCTGACTAATTGAGATGCATGCTTTGCATACTTCTGCCT-- +Tau (WT)		1.00	1.00
-----TGTTGCTGACTAATTGAGATGCTTTGCATACTTCTGCCT-- -4		0.69	0.92
-----TGTTGCTGACTAATTGAGATGCAGATCTGCTTTGCATACTTCTGCCT-- +4		1.14	NT
-----TGTTGCTGACTAATTGAGATGCCAAGCTTGGCTTTGCATACTTCTGCCT-- +6		0.26	0.28
TGTTGCTGACTAATTGAGATGGCTTAATTAATTAAGCCTTTGCATACTTCTGCCT-- +12		0.36	0.18

*-Log % Bound/fmol competitor

FIG. 8. Results of binding competition analyses. Binding of the crude (Nuc. Ext.) or APF Band A factors to the +Tau probe (WT) was competed by using fragments analogous to +Tau but containing altered sequences as shown. Competition curves were generated, and the competitive efficiency was calculated (see text) relative to the efficiency of self competitor (i.e., competition using +Tau competitor). The positions of the AP-1, OBP I, and OBP II binding sites in each competitor are indicated. The x marks indicate where the sequence at the 5' or 3' ends of the OBP I or II sites are altered due to construction. The -Tau competitor is cleaved at the *Sph*I site and has no sequences upstream. Although the OBP I site is nearly intact, the -Tau competitor cannot compete; apparently it cannot bind factor because of an end effect.

tion studies have indicated that the TABS in the late proximal 72-bp repeat appear to have the greatest effect on late promoter activation (34), we and others have reported effects mediated by deletions in the Ori region which affect the Ori TABS (11, 34). In addition, given the location of the three TABS in the enhancers and near Ori, it is quite possible that the Band A factor binding may affect early transcription and viral DNA replication, in addition to late transcription. In support of this argument, it has recently been reported that T/t antigen may activate transcription in the early direction (47).

The overlap of the TABS and the OBP I and OBP II sites suggests that the Band A factors may be related to OBP100/

Oct 1. However, the sizes of the two proteins are quite different, 100 kilodaltons for OBP100/Oct 1 and 20 to 28 kilodaltons for Band A factors. Since Band A factors are small, one could argue that they may represent specific proteolytic breakdown products of OTB100/Oct 1. Although we cannot eliminate this possibility, we have tested it by preparing extracts in the presence of a variety of protease inhibitors, with no significant changes in the apparent size of the Band A factors. If the Band A factors are breakdown products of OTB100/Oct 1 (or some other binding factor), our studies are still valid, for the fragments retain binding activity as well as regions which reflect modifications which occur in the presence of T/t antigen. We are continuing to

FIG. 9. Compilation of results and correlation with other data. The Tau-Delta region (see Fig. 1) is outlined, showing factor-binding sites, in particular the AP-1 site and sites I and II for OBP100/Oct 1. Also noted are domains I and II (18; see text for details). Our footprinting data for Band A factors is indicated over the AP-1, OBP II, and OBP I sites, which define a TABS (Fig. 1). Domain II corresponds exactly with the TABS. Within Domain II base substitution mutagenesis was performed to determine the effect on late transcriptional activation (40); the effects of these mutations are shown as the percent loss of activity caused by the mutations (see text for details). The binding region of Band B is known to start beyond nucleotide 220 (data not shown) and does not overlap the Band A factor site.

address the question of the integrity of the Band A factor and its possible relation to OPB100/Oct 1.

We have argued previously (4, 20) that transcriptional activation mediated by T/t antigen does not require interaction of large T antigen with DNA (small t antigen is not a DNA-binding protein). Instead T/t antigen appears to cause the modification or induction of cellular factors, e.g., the Band A factors. T/t antigen may act directly on the factors. Conversely, it may function at a greater distance, interacting with cellular processes which mediate either the activation of existing factors or the induction of new members of a family of factors. We favor the hypothesis that T/t antigen interacts with cellular processes which mediate the activation or induction of Band A factors. This is based on our preliminary observations (not shown) that in rapidly growing CV-1 cells, we can occasionally detect the modified forms of Band A factors which we routinely detect in COS cells. This suggests that the process for forming the modified factors is present in normal cells but is under rigid growth or cell cycle control. Hence the effect of T/t antigen may be to remove this control. Overall this would imply that the activation or induction of cellular transcription factors by T/t antigen would result in the alteration of cellular as well as viral gene expression. It is well known that the introduction of T/t antigen into cells has a dramatic effect on cellular gene expression, and we have previously shown that T antigen is a promiscuous activator of many viral and cellular promoters (3). This function of the SV40 early proteins may be significant in the establishment of the transformed phenotype in nonpermissive cells as well as for the induction of cellular replicative enzymes in permissive cells, which is vital for the amplification of the viral genome during the lytic cycle. Activation of the late promoter by T/t antigen may then be secondary to activation of the cellular gene expression necessary for replication.

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