Nuclear activity from F9 embryonal carcinoma cells binding specifically to the enhancers of wild-type polyoma virus and PyEC mutant DNAs

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ABSTRACT

Although wild-type polyoma virus does not productively infect murine embryonal carcinoma (EC) cells, a number of mutants (PyEC mutants) that do infect undifferentiated EC cells have been isolated. All PyEC mutants have DNA sequence alterations within the enhancer region of the viral genome. This report describes an activity present in nuclear extracts of F9 EC cells which, by "footprint" analyses, binds specifically to a small region of about 20 base pairs (nucleotides 5180-5200) within the subregion of the polyoma enhancer designated as the B or β element. While no difference in binding of factor was detected between wild-type polyoma enhancer and the enhancers of the PyEC mutants, PyF111 and PyF441, which had been selected for productive infection of F9 cells, definite differences between wild-type and mutants were observed in the digestion patterns of their naked DNAs with either DNAase I or exonuclease III. This difference was restricted to the region around the point mutation (nucleotide 5258) common to these mutant DNAs.

INTRODUCTION

Enhancers are operationally defined as DNA sequences that stimulate transcription of cis-linked genes in a manner relatively independent of location and orientation (1). Sequences having enhancer activity have been found in a number of viral genomes including, but not limited to, those of simian virus (SV) 40 (2-5), polyoma virus (6), and bovine papilloma virus (BPV; 7,8). Enhancer sequences have also been identified in some eukaryotic cellular genes (9-12). Several studies suggest that enhancers are involved in cell type-specific gene expression, either with respect to species (4,13-15) or to differentiation state (9,10,12,16), but more recent evidence (17-23) indicates that enhancer sequences by themselves may not be sufficient for cell type-specific control of transcription. It appears that the cooperative interaction of several genetic elements must occur for regulated gene expression. A number of observations (24-28) suggest the interaction of trans-acting factors with enhancer sequences, but no factor exhibiting cell type specificity with respect to enhancer function has yet been isolated. Thus,

the nature of trans-acting factors interacting with enhancer sequences has not been well characterized, and the mechanism by which enhancers affect specific transcription remains a mystery.

The enhancer region of the polyoma virus genome is located within a 246 base pair (bp) segment between the Bcl I site at nucleotide 5046 (using the numbering system of Deininger *et al.*, 29) to the Pvu II site at nucleotide 5292 (6). Work from several laboratories has indicated that this enhancer region can be divided into at least two subregions which apparently can function independently of each other (30-32). One subregion, designated A or α , is located within the 109 bp fragment from the Bcl I site to the Pvu II site at nucleotide 5155; a second subregion, designated B or β , is located within the 137 bp Pvu II to Pvu II (Pvu II-4) fragment. In addition to its role in transcription, the polyoma enhancer is required for viral DNA replication (33-35). As in the case of transcription, at least two subregions can be identified within the enhancer which are functional with respect to DNA replication (35-37).

Mutations within the polyoma enhancer can affect the host range properties of the virus. Wild-type polyoma does not productively infect murine teratocarcinoma stem cells, called embryonal carcinoma (EC) cells (38). Differentiation of EC cells leads to cell types that are permissive for polyoma infection (38-41). Several host range mutants of polyoma that infect undifferentiated EC cells have been isolated and characterized (42-47). All of these mutants, designated PyEC mutants, have DNA sequence alterations within the polyoma enhancer. Other host range mutants of polyoma that infect murine trophoblast cells (48), murine neuroblastoma cells (49), or Friend erythroleukemia cells (50,51) have been described. All of these mutants also have sequence alterations within the enhancer.

An interesting property of these polyoma host range mutants is that they have rather stringent cell type specificities. Thus PyEC mutants isolated for growth on PCC4 EC cells do not infect F9 EC cells; similarly, mutants isolated for growth on F9 cells do not efficiently infect PCC4 cells (43,46,52). This difference in cell type specificity is reflected by differences in the nature of the mutations present in these two groups of PyEC mutants. The PyEC(F9) mutants are usually characterized by an A:T to G:C transition at nucleotide 5258, often accompanied by tandem duplications of sequences encompassing the point mutation (42,43,46). These mutations are located within the Pvu II-4 fragment of polyoma DNA which includes the B/ β element of the polyoma enhancer. In contrast, The PyEC(PCC4) mutants are characterized by deletion of

sequences within the Pvu II-4 fragment, accompanied by duplication and translocation of sequences within the A/ α element of the polyoma enhancer (44). Polyoma mutants isolated on murine trophoblast cells differ from the PyEC mutants by having deletions in the region between the A/ α and B/ β elements (48). Polyoma mutants selected on murine neuroblastoma and Friend cells contain direct tandem duplications of varying lengths within the A/ α element (49-51). Duplication of enhancer sequences per se does not necessarily lead to altered host range properties as several natural variants of polyoma virus with wild-phenotype contain tandem duplications of sequences within the enhancer (53).

Because specific DNA sequence alterations within the polyoma enhancer affect the host range properties of the the virus, this laboratory has been searching for factors in EC cells that specifically interact with the enhancer region of polyoma DNA. The rationale for this search is that identification and characterization of such factors could provide information relevant to regulatory mechanisms affecting viral gene expression or DNA replication, which in turn may be relevant to mechanisms related to cellular gene expression or DNA replication during growth and differentiation of EC cells. I describe an activity present in nuclear extracts of F9 EC cells that specifically binds to the enhancer region of polyoma DNA. Analyses by filter binding and by "footprinting" with DNAase I and with exonuclease (Exo) III map the binding site to a small region within the B/ β element of the polyoma enhancer. The activity binds to the DNAs of wild-type polyoma and the PyEC mutants, PyF111 and PyF441, which had been selected for growth in undifferentiated F9 cells, and thus, cannot account for the host range specificity of these mutants. However, nuclease digestion analyses of naked DNAs suggest an inherent structural difference between wild-type and PyF441 DNAs that can be distinguished by either DNAase I or Exo III. This structural difference is located in the region around the PyF441 point mutation in the B/ β element of the polyoma enhancer.

MATERIALS AND METHODS

Cells and Viruses

Methods for culture and infection of mouse F9 EC cells and 3T6 cells, and wild-type polyoma strain A3 and the PyEC(F9) mutants, F101, F111 and F441, were described previously (42). PyF441 has a point mutation at nucleotide 5258. PyF101 and PyF111 have tandem duplications of 54 and 31 bp, respectively, of sequences encompassing the point mutation with both copies of the duplications containing the point mutation.

Buffers and Solutions

TBS is 140 mM NaCl, 5 mM KCl, 7 mM Na₂HPO₄, 0.1% glucose, 50 mM Tris, adjusted to pH 7.4 with HCl. Lysis buffer (54) contains 10 mM HEPES, 0.5 mM spermidine, 0.15 mM spermine, 1 mM EDTA, 0.25 mM EGTA, 500 mM sucrose, 50 mM NaCl, 7 mM 2-mercaptoethanol, 0.003 TIU/ml aprotinin, 0.5 mM PMSF, adjusted to pH 8 with NaOH. Extraction buffer is identical to lysis buffer except that 10% glycerol is substituted for sucrose. Tris-acetate buffer is 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, adjusted to pH 7.8 with acetic acid. TBE (55) is 89 mM Tris, 89 mM boric acid, 2 mM EDTA.

Preparation of Nuclear Extracts

F9 cells were grown in 15 cm plates to a density of $2-5 \times 10^7$ and washed once with cold TBS. All further procedures were performed at 0-4⁰. The cells were harvested by scraping with a rubber policeman into TBS and collected by centrifugation at 2000xg for 10 min. The cells were washed by suspension and centrifugation in lysis buffer, then suspended in lysis buffer containing 0.05% NP-40. Cell lysis was monitored by phase microscopy. Upon nearly complete release of nuclei, the lysate was centrifuged at 3000xg for 10 min. The nuclei were extracted by a modification of the procedure of Borgmeyer *et al.* (54). The nuclear pellet was washed with extraction buffer, then suspended in extraction buffer at a concentration of approximately 10^8 nuclei/ml. An equal volume of extraction buffer containing 0.55 M NaCl was added slowly, and the mixture, now containing 0.3 M NaCl, was left on ice for 30 min with occasional shaking. Extracted nuclei were removed by centrifugation at 10,000xg for 20 min, and the supernatant was passed through a column of DEAE cellulose equilibrated with extraction buffer containing 0.3 M NaCl. The eluate from the DEAE cellulose column was treated with solid ammonium sulfate to give 70% saturation. Precipitated proteins were centrifuged and dissolved in extraction buffer containing 0.05 M NaCl at a concentration of 1-4 mg/ml as determined by the method of Bradford (56) using a protein assay kit from BioRad with bovine serum albumin as standard. Aliquots of this preparation, called F9 nuclear extract, were frozen on dry ice and stored in liquid nitrogen or in a -90⁰ freezer. The preparation of 3T6 nuclear extract was identical to that described for F9 nuclear extract except that the 3T6 cells were lysed by suspension in lysis buffer containing 0.5% NP-40.

Nitrocellulose Filter-Binding Assay

Supercoiled polyoma virus DNA, purified by CsCl centrifugation as before (42), was restricted by appropriate restriction enzymes, then end-labelled with ³²P at the 5' end using T4 polynucleotide kinase or at the 3' end using

the Klenow fragment of DNA polymerase I by standard methods (55). End-labeled fragments (ca. 10 ng) were mixed with varying amounts of nuclear extracts in binding mixture (100 μ l) containing 10 mM HEPES (adjusted to pH 8.0 with NaOH), 2 mM DTT, 5 mM MgCl₂, 0.1 mM EDTA, 100 mM NaCl, and the amounts of sheared calf thymus DNA indicated for individual experiments. The reactions were kept on ice for at least 1 hr, then passed through nitrocellulose filters (Schleicher and Schuell, BA85). The filters had been washed in H₂O, boiled for 10 min in H₂O, then soaked in binding buffer. Samples were passed through either 2.5 cm filters in a standard suction apparatus or individual wells of a Schleicher and Schuell Minifold dot-blot apparatus. The filters were then washed with three aliquots (0.5 ml each) of binding buffer. DNA fragments retained on the filter were eluted overnight at room temperature with 0.3 ml of 0.5% SDS, 10mM Tris adjusted to pH 7.4 with HCl, 1mM EDTA, 100 μ g/ml proteinase K. The eluted fragments were extracted twice with phenol:chloroform (1:1), once with chloroform, then precipitated with ethanol in the presence of 20 μ g of yeast RNA as carrier. Fragments were dissolved in sample buffer containing 1% Ficoll, 50 mM EDTA, and 0.01% each bromophenol blue and xylene cyanole FF, then subjected to electrophoresis in agarose or polyacrylamide gels using Tris-acetate buffer. Gels were dried, then exposed against Kodak XAR-5 film, usually at -70⁰ using Dupont Cronex Lightning Plus screens.

Footprint Analysis

For protection experiments, the Bcl I to Msp I (nucleotide 24) fragment of polyoma DNA was labeled at the 5'-end of the early strand at the Bcl I site (E strand probe) or at the 5'-end of the late strand at the Msp I site (L strand probe). The E strand probe was prepared by restriction of polyoma DNA with Bgl I and Bcl I, followed by agarose gel electrophoresis. The smaller Bcl I/Bgl I fragment was located by ethidium bromide staining and electroeluted onto DEAE nitrocellulose (Schleicher & Schuell, NA-45). The fragment was recovered from the NA-45 as prescribed by the manufacturer and labeled with polynucleotide kinase and γ -³²P-ATP by standard methods (55). The labeled fragment was digested with Msp I, and the Bcl I to Msp I subfragment was isolated by agarose gel electrophoresis and electroelution onto DEAE nitrocellulose as described above. The L strand probe was prepared by similar methods from the Msp I-3 fragment, which after labeling was cleaved with Bcl I.

DNAase I footprinting (57) was performed on binding reactions (50 μ l) prepared as described above for filter binding assays except that they contained 1-10 ng of E or L strand probe and 1-2 μ g of sheared calf thymus

DNA. After incubation for at least 30 min on ice, the reactions were brought to room temperature for 5 min. Five μ l of an appropriate dilution (2-20 μ g/ml) of DNAase I (Worthington DPFF, freshly diluted from a 1 mg/ml stock into dilution buffer containing 20 mM Tris-HCl pH 7.4, 50 mM KCl, 5 mM MgCl₂, 3 mM CaCl₂, 20% glycerol) was added and the mixture was incubated for 30 sec at room temperature. Reactions were terminated by the addition of 100 μ l of 1% SDS, 50 mM EDTA pH 8.0, 20 μ g yeast RNA. After organic extraction as above, nucleic acids were precipitated with ethanol, washed with 70% ethanol, dried, and dissolved in 5 μ l of 90% formamide, 0.5xTBE, 0.01% each bromophenol blue and xylene cyanole FF. Samples were analyzed by electrophoresis in 0.4 mm-thick sequencing gels containing 5% polyacrylamide and 8 M urea using TBE buffer. Gels were dried and exposed against X-ray film as above.

Exo III protection footprinting was performed by a modification of the method of Wu (58). Binding reactions (50 μ l) contained 15 mM Tris-HCl pH 7.4, 0.5 mM DTT, 5 mM MgCl₂, 0.1 mM EGTA, 75 mM NaCl, 5% glycerol, 2 mM Na phosphate pH 7.2, 200 μ g/ml *E. coli* tRNA, 40 μ g/ml sheared calf thymus DNA, ³²P-end labeled E or L strand probe as above, and various amounts of nuclear extract. After incubation on ice for at least 1 hr, appropriate amounts of Exo III (Boehringer-Mannheim, 100 units/ μ l) were added, and the reactions were incubated for 10 min at 30°. Reactions were terminated and analyzed as described above for DNAase I footprinting.

RESULTS

Nitrocellulose Filter Binding

End-labeled Msp I fragments of wild-type polyoma and PyF441 DNAs were incubated with F9 nuclear extract in the presence of different amounts of unlabeled calf thymus DNA, then passed through a nitrocellulose filter. DNA fragments retained on the filter were eluted and analyzed by agarose gel electrophoresis. The results shown in Figure 1a indicate that in the absence of calf thymus DNA, no specificity in retention of DNA fragments was observed. As increasing amounts of calf thymus DNA were added to the binding reaction, the relative amount of Msp I fragment 3 retained on filters was enriched with respect to the other fragments. The Msp I-3 fragments of both wild-type and F441 DNAs were retained on filters with F9 nuclear extract in the presence of calf thymus DNA. A similar pattern of fragment retention was observed for 3T6 nuclear extract, and no fragments were retained on filters in the absence of nuclear extract (see lanes "-" in Fig. 1b) or in the presence of a comparable amount of bovine serum albumin (data not shown).

Figure 1. Retention of specific fragments of polyoma DNA on nitrocellulose filters by F9 nuclear extract. DNA fragments of wild-type (WT) and PyF441 DNAs were 5' end-labeled using γ - ^{32}P -ATP and T4 polynucleotide kinase, incubated with 2 μg (a) or 0.5 μg (b) F9 nuclear extract in the presence of 0 (A), 0.01 (B), 0.1 (C), 1 (D), or 10 (E) μg of sheared calf thymus DNA, then passed through nitrocellulose filters as described in Materials and Methods. Fragments retained on filters were eluted and analyzed by electrophoresis in agarose gels. Marker (M) lanes show the total pattern of labeled fragments present in the binding reactions. Panel (a) shows results of binding with total Msp I fragments (ca. 10 ng per assay) of polyoma DNA fractionated in a 1.5% agarose gel. Panel (b) shows results of binding with the two subfragments (ca. 1 ng per assay) of polyoma DNA generated by cleavage of the Msp I-3 fragment with Bcl I analyzed by electrophoresis in a 2% agarose gel. Fragments retained on filters in the absence of nuclear extract were analyzed on lanes marked (-).

Cleavage of the 885 bp Msp I-3 fragment of polyoma DNA with Bcl I yields two fragments of 612 bp and 273 bp. Nitrocellulose filter binding assays indicated that the smaller fragment was selectively retained on filters in the presence of F9 nuclear extract and competing calf thymus DNA (Figure 1b), localizing the binding site(s) between nucleotides 5046 and 24.

To map the binding site(s) further, the 746 bp Bam HI to Bgl I fragment of polyoma DNA was end-labeled, separately digested with Msp I, Pvu II or Bcl I, then the intact and digested fragments were mixed in equal molar amounts and tested for binding with nuclear extracts. The results, shown in Figure 2, illustrate that selective binding occurred only with DNA fragments containing the sequences between the Pvu II site at nucleotide 5155 and the Msp I site at nucleotide 24. This region includes the Pvu II-4 fragment plus 27 bp. Very similar results were obtained for wild-type and F441 DNA fragments.

Protection Footprinting

To map the sequences that interact with the binding activity in F9

Figure 2. Localization of binding site by nitrocellulose filter-binding assay. The 746-bp Bam HI to Bgl I fragments of wild-type (WT) and PyF441 DNAs were end-labeled using α - 32 P-dGTP and the Klenow fragment of DNA polymerase I. Both 3' ends were labeled under the conditions used. Labeled fragments were digested with Msp I, Pvu II, or Bcl I. Approximately 1 ng of an equimolar mixture of these fragments was incubated with 0.5 μ g (WT) or 1 μ g (F441) of F9 nuclear extract, passed through nitrocellulose filters, and retained fragments were analyzed by electrophoresis in 5% polyacrylamide (acrylamide:bis = 20:1) gels. The binding reactions contained 0 (A), 0.1 (B), 1 (C), or 10 (D) μ g sheared calf thymus DNA. Marker (M) lanes show the pattern of total labeled fragments present in the binding reactions. The lower panel shows the map of polyoma DNA fragments corresponding to the labeled bands in the gels.

nuclear extract, nuclease protection experiments were performed. The early (E) strand of polyoma DNA from the Bcl I site to the Msp I site at nucleotide 25 was 5' end-labeled at the Bcl I site, and the late (L) strand of this fragment was separately 5' end-labeled at the Msp I site. These fragments were reacted with nuclear extract, then treated with either DNAase I or with Exo III. Protected sequences were determined by electrophoresis on sequencing gels with control fragments treated with either DNAase I or Exo III without prior reaction with nuclear extract.

DNAase I Protection

Figure 3 shows the DNAase I footprints of the L strand of wild-type and PyF441 DNAs. Unexpectedly, the pattern of DNAase I cleavage obtained for naked DNA differed between the wild-type and mutant DNAs (compare lanes A in the region around the solid triangle in Fig. 3). This difference occurred at the site of the point mutation in PyF441 DNA, suggesting that the wild-type and mutant DNAs have an inherent difference in structure near the mutated site. Such structural differences can apparently result from just a point mutation. The DNAase I cleavage patterns of the naked DNAs of PyF101 and PyF111 around the site of the point mutation were identical to that of PyF441 (data not shown). The difference between wild-type and mutant footprint patterns

Figure 3. DNAase I footprint analysis of the L strand of polyoma DNA. The L strand probe was 5' end-labeled at the Msp I site as described in Materials and Methods. The probe was incubated with 0 (A), 27 (B), or 54 (C) μ g of F9 nuclear extract, then reacted with 10 (A), 50 (B), or 100 (C) ng of DNAase I. Resulting DNA fragments were analyzed by electrophoresis in 5% polyacrylamide, 8 M urea sequencing gels. The same probe was incubated with 0 (E) or 54 (D) μ g of F9 nuclear extract, reacted with 100 (E) or 200 (D) units of Exo III, and analyzed in the same gel. The locations of the Bcl I and Pvu II sites, the homologies to the SV40 and BPV core enhancer sequences, the 9 bp inverted repeat (arrows), and the site of the PyF441 point mutation (solid triangle) are indicated with respect to the gel pattern (see text for details). The solid bar indicates the region protected from digestion by DNAase I.

remained unchanged in the presence of F9 nuclear extract, and no obvious protection of sequences around the mutation was observed. However, one band corresponding to cleavage between nucleotides 5272 and 5273 did appear to be

Figure 4. DNAase I footprint analysis of the E strand of polyoma DNA. The E strand probe was 5' end-labeled at the Bcl I site as described in Materials and Methods. The details of the experiment are identical to those described in the legend for Figure 3. Reactions contained 0 (A), 27 (B), or 54 (C) μ g of F9 nuclear extract and were digested with 10 (A), 50 (B), or 100 (C) ng of DNAase I. Also shown is the analysis of E strand probe incubated with 0 (E) or 54 (D) μ g F9 nuclear extract and digested with 100 (E) or 200 (D) units of Exo III. Lane (M) contains molecular weight markers. Msp I and Pvu II restriction sites, SV40 and BPV homologies, the GC-rich palindrome (arrows), and the site of the PyF441 point mutation (solid triangle) are shown. The protected region is indicated by the solid bar.

enhanced in the presence of nuclear extract (see lower of the two BPV homologies in Fig. 3). This enhancement was observed for both wild-type and mutant DNAs. Protection due to nuclear extract was observed in an 18 base region between nucleotides 5181 and 5198 (compare lanes A with lanes B and C in the vicinity of the solid bar in Fig. 3).

Figure 4 shows the DNAase I footprint pattern of the E strand of polyoma DNAs. The results were similar to those observed for the L strand. No protection was seen for sequences around the PyF441 point mutation. Protection did occur in a 17 base region between nucleotides 5184 and 5200 (solid bar in Fig. 4). The E strand protection pattern was less clear than that for the L strand, but the protected regions on the two strands corresponded very closely to each other (see Fig. 6).

Exonuclease III Protection.

Because the DNAase I footprints had considerable amounts of background, an independent footprinting method, Exo III protection mapping (see 58 and references cited therein), was used to corroborate the results of DNAase I

Figure 5. Exonuclease III footprint analyses. The L and E strand probes of wild-type (WT), PyF111, and PyF441 were prepared as described in Materials and Methods. Probes were incubated with no nuclear extract (A), with 25 (B) or 50 (C) μ g of F9 nuclear extract, or with 35 (D) or 70 (E) μ g of 3T6 nuclear extract, then treated with 100 units of Exo III. Resulting digests were analyzed by electrophoresis in 6% polyacrylamide, 8 M urea sequencing gels. Lanes marked (M) contain molecular weight markers.

experiments. For these studies, 5' end-labeled fragments were treated with Exo III after incubation with or without nuclear extract. In the absence of nuclear extract, Exo III generates a series of limit digests. In the presence of nuclear extract, specific binding of factor to the DNA blocks Exo III, resulting in fragments differing from the limit digests.

The results of Exo III protection experiments (Figure 5) were consistent with the DNAase I footprinting results. First, there was a difference between wild-type and PyF DNAs in the pattern of digestion of the L strand by Exo III

in the absence of nuclear extract. As was the case with DNAase I, this difference occurred around the site of the PyF point mutation (compare lanes A for the L strand probes in Fig. 5). The major Exo III stop sites in the presence of nuclear extract were in the same region protected from digestion by DNAase I. These major stop sites were located within a 26 base region (nucleotides 5195-5220) on the E strand and a 22 base region (nucleotides 5178-5199) on the L strand. The stop sites were clustered in the regions mentioned, and strong stop sites were spaced at 8-10 nucleotide intervals within these regions on both strands. Similar protection patterns were observed for F9 and 3T6 nuclear extracts (compare lanes B and C with lanes D and E in Fig. 5). Very strong stop sites were observed on both the early and the late strands at the 3'-proximal side of the region protected by DNAase I (see Figures 3 and 4, lanes C and D, to compare DNAase I protection pattern with Exo III protection pattern). As was the case with DNAase I, no difference in protection by nuclear extract was detected between wild-type and PyF mutant DNAs using Exo III. A summary of the DNA sequences protected by DNAase I and Exo III is shown in Figure 6.

DISCUSSION

The present results indicate the existence of an activity in nuclear extracts of F9 and 3T6 cells that binds specifically to the enhancer region of polyoma virus DNA. The binding site is located within the Pvu II-4 fragment of polyoma DNA within a subregion of the polyoma enhancer designated as the B/β (30,35) element. The DNAase footprint covers an inverted repeat of the 6-bp sequence 5'-AGTTC (nucleotides 5183-5188 and 5192-5197 in Fig. 6). Several other structural features of the polyoma genome exist very close to this binding site. These include a GC-rich palindrome, a core sequence identified by Weiher *et al.* (59) to be crucial for activity of the simian virus (SV) 40 enhancer, and one of two sites in polyoma chromatin that are hypersensitive to digestion by DNAase I (60). The importance of these DNA sequences for polyoma infection of F9 cells has not been established, but it may be noteworthy that the A2 and A3 strains of polyoma differ by two nucleotide pairs in the GC-rich palindrome, the A2 DNA lacking the T:A base pair at nucleotide 5197 and the A:T base pair at nucleotide 5212 (61). The positions of these two nucleotide pairs in A3 DNA (29) are such that they preserve the palindromic structure (see Fig. 6), arguing for its importance during viral infection. However, it is known that deletion of this region of the polyoma genome does not eliminate the viability of the virus in mouse fibroblasts

(31,36,37). This apparent discrepancy could indicate that different elements of the polyoma enhancer are functionally redundant in some cell types but essential in others.

No clearcut binding was detected for the region around the site of the PyF441 point mutation at nucleotide 5258. This point mutation is important for polyoma infection of F9 EC cells (42,46) and is located between two copies of a direct repeat of 8 bp. The sequences in the direct repeat are similar to repeated sequences observed by Lusky *et al.* (8) to be present in the BPV and the SV40 enhancers. Furthermore, the binding site observed here is located outside of the sequences that are tandemly duplicated in F101 and F111 mutant DNAs (see Fig. 6). Thus, the activity described does not appear to bind to DNA sequences specific for these PyEC(F9) mutants, at least under conditions used here. On the other hand, cleavage by DNAase I at one site 15 nucleotides from the PyF441 point mutation was enhanced in the presence of F9 nuclear extract. Whether this is due to specific interaction of some factor(s) with these DNA sequences is not known.

Although no difference in binding was detected between wild-type and PyEC mutant enhancer sequences, a clear difference between these two DNAs was seen in either DNAase I or Exo III digestion patterns in the absence of nuclear extract. This difference in pattern was located around the PyF441 point mutation, suggesting that this region of mutant and wild-type DNAs may have conformational differences resulting from a single base pair transition. Conformational alteration of naked DNA due to deletion of three nucleotide pairs has been reported for a region upstream of the promoter for a tRNA operon in *Salmonella* (62). This mutation caused reduced transcription of the operon, suggesting a functional role of DNA conformation in gene expression. It is tempting to speculate that conformational features in DNA can be distinguished by nuclear factor(s) in F9 cells leading to differential expression of wild-type and mutant genomes in these cells. Alternatively, the differences in structure between wild-type and mutant enhancers observed in linear DNA under these *in vitro* conditions may induce additional structural differences *in vivo* (for example, under torsional stress in supercoiled molecules; 63). These induced structural features could be recognized by nuclear factor(s).

A recent paper by Piette *et al.* (64) describes an activity in 0.3 to 0.55 M NaCl extracts of 3T6 nuclei that binds specifically to the B/ β element of the wild-type polyoma enhancer. This activity apparently consists of at least two factors, and DNAase I footprinting showed that the activity protects about

55 nucleotides (nucleotides 5160 to 5215 in Fig. 6) of the E strand and about 30 nucleotides (5190 to 5220) of the L strand. The protection patterns are similar but not identical to those described here for the F9 binding activity. No evidence of multiple factors is inferred from this work, and the size of the DNAase I footprint (about 20 nucleotides on both strands) suggests the binding of a single factor molecule. As the F9 nuclei were extracted with 0.3 M NaCl, it is possible that some factors extracted by Piette *et al.* (64) from 3T6 nuclei were not present in the F9 nuclear extracts. Alternatively, F9 and 3T6 nuclear extracts may contain factors which, while binding to the same region of the polyoma genome, are different from each other. Perhaps relevant is the observation that the 0.3 M NaCl nuclear extract of 3T6 cells, although behaving similarly to F9 nuclear extract with regard to the nitrocellulose filter-binding assay and Exo III footprinting using polyoma DNA, has not yielded a clear DNAase I footprint (unpublished).

How specific sequence alterations within the polyoma enhancer can lead to different host range properties is not known. As mentioned earlier, one possibility is the existence of cell type-specific trans-acting factors that bind to specific enhancer sequences. The enhancer-binding activity described here apparently does not show the specificity required of such a cell type-specific factor. At the same time, the observation that a point mutation in PyF441 DNA can cause changes in the cleavage pattern of DNAase I and the stop sites of Exo III in naked DNA when compared to wild-type polyoma DNA provides some basis for a mechanism involving interaction of cell type-specific factors with specific enhancer sequences. Of course, a binding assay alone is insufficient to elucidate the role(s) of putative trans-acting factors in regulation of gene expression. To identify and characterize these factors, it is necessary to develop an independent functional assay to carry out in parallel with binding studies. Only then can one begin to assess the possible functional role(s) of trans-acting factors in enhancer activity.

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