Interaction of a nuclear factor with the polyomavirus enhancer region

(DNA binding protein/eukaryotic enhancer element)

P. OSTAPCHUK*, J. F. X. DIFFLEY†, J. T. BRUDER*, B. STILLMAN†, A. J. LEVINE‡, AND P. HEARING*§

*Department of Microbiology, Health Sciences Center, State University of New York, Stony Brook, NY 11794; †Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; and ‡Department of Molecular Biology, Princeton University, Princeton, NJ 08544

ABSTRACT   We have identified a factor present in nuclear extracts of undifferentiated F9 murine embryonal carcinoma cells that specifically interacts with the polyomavirus enhancer region. Nuclease "footprint" analysis was used to define the binding site that corresponds precisely to the boundaries of polyoma enhancer element C defined by Veldman et al. [Veldman, G. M., Lupton, S. & Kamen, R. (1985) Mol. Cell. Biol. 5, 649–658] that is required as an enhancer for efficient viral DNA replication and early and late region transcription. The region of nuclease protection contains a 6-base-pair inverted repeat, separated by 3 base pairs, and symmetrical flanking DNase I hypersensitive cleavage sites, suggesting that this factor may bind as a dimer. A cloned 29-base-pair polyoma DNA fragment contains an intact binding domain. Similar levels of binding activity were found in nuclear extracts prepared from differentiated murine F9 cells, as well as murine L cells and human HeLa cells. The factor has been termed "EF-C" for enhancer binding factor to polyoma element C.

Eukaryotic enhancer sequences regulate a variety of viral and cellular genes (reviewed in refs. 1 and 2). By definition, enhancers activate transcription of a gene linked in cis in a relatively orientation- and location-independent fashion. Enhancer elements have been shown to display tissue and species specificity and to activate transcription over large distances (1, 2). Although the mechanism(s) of enhancer function is unclear, these sequences have been shown to bind trans-acting cellular factors (3–5).

We are interested in host factors that bind to the murine polyomavirus enhancer region. The polyoma enhancer is particularly interesting because this region regulates viral expression in a developmentally specific fashion (see ref. 6 for a review) and also is required for efficient viral DNA replication (7). deVilliers and Schaffner (8) originally identified an enhancer located in the noncoding sequences of the polyoma genome [nucleotides (nt) 5021–5262, Fig. 1]. This region has an altered chromatin structure (17) and contains control elements for the early and late transcription units and the origin of DNA replication (refs. 7, 8, and 17–22; Fig. 1). Genetic analyses have demonstrated that the organization of the polyoma enhancer is complex and functionally redundant (although not sequence redundant). At least five separate domains appear to contribute to the enhancer function of this region (regions A–E, Fig. 1; refs. 7 and 14–16; also see ref. 6 for a review). The multiple nature and functional redundance of the polyoma enhancer sequences may reflect a capability to utilize a variety of cellular factors to obtain optimal enhancer activity.

Polyomavirus lytic growth is restricted in a number of undifferentiated mouse cell lines that include the embryonal carcinoma lines F9 and PCC-4 as well as trophoblast and neuroblastoma cell lines. Polyoma host range mutants adapted for growth in these undifferentiated cells contain complex point and deletion mutations, duplications, and rearrangements in the enhancer region (reviewed in ref. 6). The genetic alterations in these mutants confirm the importance of the enhancer regions discussed above (regions A–E, Fig. 1). Our studies have focused on the enhancer region of wild-type (wt) polyoma and one prototypic F9 host range mutant (hrN2; ref. 23). Utilizing a sensitive filter binding assay to detect DNA–protein complexes (24), we have identified a factor present in undifferentiated F9 cell nuclear extracts that binds both wt and hrN2 enhancer regions. This factor also was found in nuclear extracts from differentiated F9 cells, murine L cells, and human HeLa cells. Nuclease protection experiments showed that this factor binds precisely within the boundaries of enhancer element C (Fig. 1).

METHODS

Preparation of Nuclear Extract. Isolated nuclei (25) were washed in lysis buffer minus Nonidet P-40, and nuclear proteins were extracted using 0.3 M NaCl as described (26) with the exception that the 0.1 M NaCl wash was omitted. Extracts were dialyzed for 4 hr at 4°C against 20 mM Tris·HCl, pH 7.4/50 mM NaCl/25% (vol/vol) glycerol/0.1 mM EGTA/0.1 mM EDTA/0.5 mM dithiothreitol/phenylmethylsulfonyl fluoride at 85 μg/ml and stored in liquid nitrogen. Protein concentrations were determined by the method of Bradford (27).

Plasmids and Preparation of Labeled DNA. The Pvu II fragment 4 (nt 5128–5262) from wt Toronto strain [134 base pairs (bp)] or mutant hrN2 (167 bp; ref. 23) was inserted into the Sma I site of the vector pUC9. Cloned fragments were labeled at the EcoRI site in the pUC9 polylinker region using Klenow polymerase and [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq), excised from the plasmids by digestion with BamHI, and purified by electrophoresis. Specific activities of approximately 7.5 × 10^7 cpm/μg of DNA were obtained. Nonspecific DNAs for binding studies were the pBR322 EcoRI–EcoRV fragment (185 bp) and a pool of Hpa II fragments of equivalent size labeled in the same fashion.

Plasmid pUC-EFC (containing a minimal binding domain) was constructed by inserting an Xho I linker at the Hae III site at polyoma nt 5174 in a pUC clone containing the hrN2 Pvu II fragment 4. The plasmid was linearized adjacent to the Pvu II site at polyoma nt 5128, digested briefly with exonuclease III and then with S1 nuclease, repaired using Klenow polymerase, and Bcl I linkers were added. The DNA was digested with Bcl I and Xho I, and fragments containing the binding site for the enhancer binding factor to polyoma element C (EF-C) were inserted in pUC9 via the BamHI and

Abbreviations: bp, base pair(s); wt, wild type; nt, nucleotide(s); EF-C, enhancer binding factor to polyoma element C.
§To whom reprint requests should be addressed.
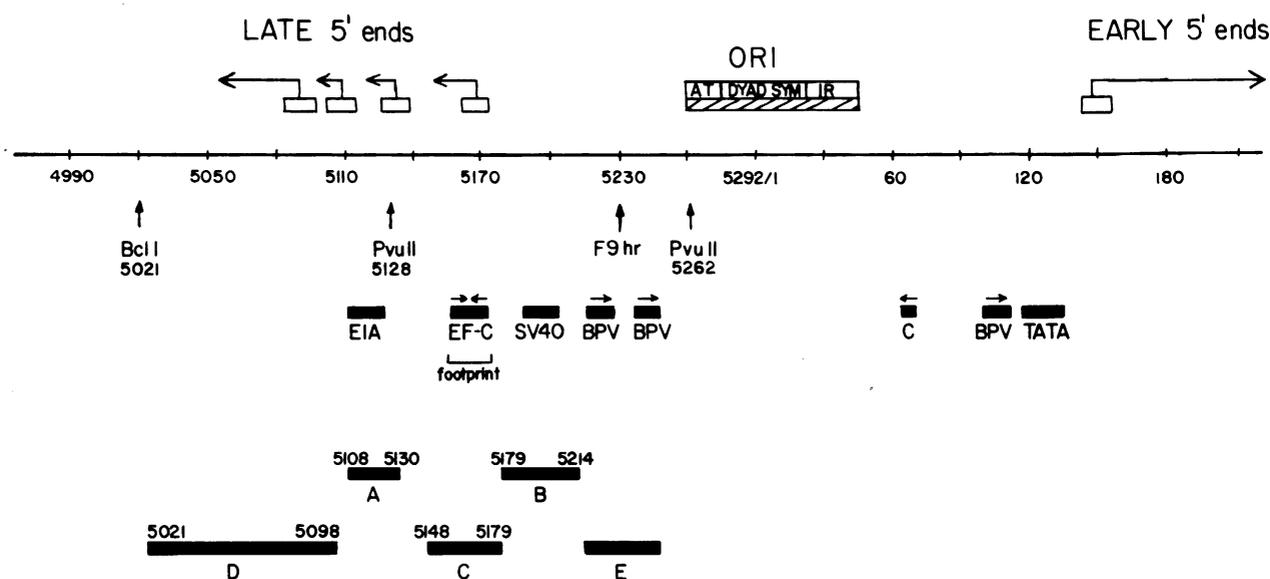
FIG. 1. A schematic drawing of the control region of polyomavirus. Nucleotide numbers of the sequenced polyoma A2 strain (9) are shown. The sequence of the polyoma A2 strain in this region is very similar to the Toronto strain used in these studies with the exception of several nucleotide changes (P.O. and P.H., unpublished data) and a duplication of nt 5096–5139 (10). Late and early region transcripts are indicated by open boxes and arrows. Ori, minimal origin of replication. Homologies to other viral enhancer regions are indicated: E1A, adenovirus E1A enhancer homology (11); SV40, homology with the simian virus 40 (SV40) core enhancer element (12); BPV, bovine papilloma virus enhancer homology (13). EF-C is the region of nuclease protection described in this report, and the arrows indicate repeated sequences in this element. C, single copy of the repeat in the EF-C binding domain; TATA, TATA box promoter homology. A, B, C, and D indicate elements of the polyoma enhancer defined by Veldman *et al.* (14). Region E corresponds to an additional enhancer region generated in polyoma F9 host range mutants (15, 16).

Sma I (converted to *Xho* I via a linker) sites. The clone displayed in Fig. 5 (pUC-EFC) was selected for these studies.

Filter Binding Assay and DNase I "Footprint" Analysis. The filter binding assays (50 μl, final reaction volume) were performed according to the procedure of Diffley and Stillman (24). Various concentrations of the competitor DNA [poly-(dA)·oligo(dT)/poly(dC)·oligo(dG) homopolymer mix] were incubated with nuclear extract in 25 mM Hepes·KOH, pH 7.4/5 mM MgCl/2 mM dithiothreitol/bovine serum albumin at 50 μg/ml/50 mM KCl for 15 min at room temperature. ^{32}P-labeled DNA was added (2500–6000 cpm corresponding to 33–80 pg of DNA), and the reaction was continued for 30 min at room temperature. Reactions were slowly filtered through nitrocellulose using a Schleicher & Schuell manifold. The filter was washed three times with 0.25 ml of ice cold 25 mM Hepes·KOH, pH 7.4/5 mM MgCl; and either the filter was exposed to x-ray film, or the radioactivity in the dots was measured directly by liquid scintillation counting.

Binding reactions for DNase I footprint analysis were scaled up 5-fold. Following the binding reaction, 12 μl of DNase I (Worthington; concentrations are in the figure legends) in 25 mM Hepes·KOH, pH 7.5/100 mM MgCl₂ and 50 mM CaCl₂ were added, and the reaction mixtures were incubated for 5 min at room temperature. DNase I concentrations that yielded single hit kinetics were determined empirically. Reactions were terminated by the addition of 250 μl of 100 mM Tris·HCl, pH 8.0/20 mM EDTA/0.5% NaDod-SO₄/0.15 M NaCl/tRNA at 50 μg/ml, followed by extraction with phenol/chloroform (1:1, vol/vol), chloroform, and subsequent ethanol precipitation. Samples were electrophoresed in 8% polyacrylamide/urea denaturing gels. Marker sequencing ladders were generated using homologous fragments by the procedure of Maxam and Gilbert (28).

Cell Culture. F9 thymidine kinase deficient (F9 tk⁻) embryonal carcinoma cells (29), kindly provided by Barbara Knowles (Wistar Institute, Philadelphia) were plated on gelatin-coated dishes in Delbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum. F9 cells were stimulated to differentiate with 50 nM retinoic acid

and 0.1 mM dibutyrl cyclic AMP as described (30) and were harvested 3–4 days later. Differentiation was monitored visually and by assaying plasminogen activator activity (30). HeLa cells were cultured in suspension in the suspension formulation of Eagle's modified essential medium (SMEM) containing 7% (vol/vol) calf serum, and L cells were grown on dishes in DMEM containing 10% (vol/vol) calf serum.

RESULTS

A nitrocellulose filter binding assay (24) was employed to detect factors that specifically bind to polyoma *Pvu* II fragment 4 (nt 5128–5262, Fig. 1) from the wt and the F9 hrN2 mutant. The *Pvu* II fragment 4 contains enhancer elements B, C, and E (Fig. 1). Region E is altered by point mutation (adenosine to guanosine transition at nt 5230) and subsequent duplication (nt 5214–5246) in the hrN2 mutant (18). Extracts contained proteins eluted from nuclei using 0.3 M NaCl.

The results of a filter binding assay using a nuclear extract from undifferentiated F9 cells is shown in Fig. 2. Binding of a pBR322 control fragment occurred in the absence of competitor DNA but was eliminated with increasing concentrations of competitor in the reaction. Similar results were obtained with other control fragments (data not shown). In contrast, binding of the wt or hrN2 *Pvu* II fragments 4 was observed even at high concentrations of competitor DNA. Similar titrations using nuclear extracts from differentiated F9 cells and human HeLa cells (Fig. 2) as well as mouse L cells (data not shown) also demonstrated specific binding to the wt and hrN2 *Pvu* II fragments 4. Titration experiments also were performed using increasing concentrations of nuclear extract in the binding assay with a fixed concentration of competitor DNA. With increasing concentrations of extract, binding of pBR322 DNA varied marginally above background while specific binding of the wt or hrN2 *Pvu* II fragment 4 increased to saturation (data not shown).

We used DNase I footprint analysis to define the binding site(s) for the factor(s) interacting with the polyoma enhancer fragments. Conditions for binding at saturation were used
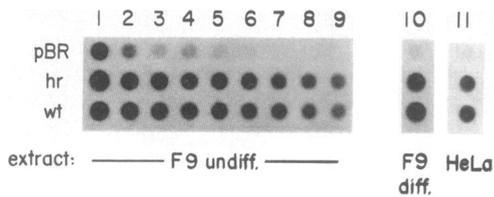
FIG. 2. Titration of competitor DNA in the filter binding assay. pBR, EcoRI–EcoRV fragment of pBR322 used as nonspecific DNA; host range (hr), *Pvu* II fragment 4 from F9 mutant hrN2; wt, *Pvu* II fragment 4 of wt polyoma virus. Binding reactions were performed as described in the text, filtered through nitrocellulose, and exposed to film for autoradiography. The 50-μl binding reactions contained 0.65 μg of protein (nuclear extract), 2000 cpm of labeled DNA, and various concentrations of competitor DNA. Lanes 1–9 contained the following concentrations of competitor DNA in binding assays using nuclear extracts prepared from undifferentiated F9 cells. Lanes: 1, 0 μg; 2, 0.0105 μg; 3, 0.0158 μg; 4, 0.0268 μg; 5, 0.0525 μg; 6, 0.105 μg; 7, 0.158 μg; 8, 0.263 μg; 9, 0.525 μg. The binding reactions in lanes 10 and 11 utilized nuclear extracts prepared from differentiated F9 cells and human HeLa cells, respectively. Lane 10, 3.5 μg of protein, 6000 cpm of labeled DNA, and 1.05 μg of competitor DNA. Lane 12, 0.6 μg of protein, 2000 cpm of labeled DNA, and 0.105 μg of competitor DNA.

followed by DNase I digestion to give single nuclease cuts per molecule. As shown in Fig. 3, identical regions of protection were detected with the wt polyoma and hrN2 fragments using nuclear extracts from undifferentiated F9 cells. The footprint is located between nt 5155 and 5174. An identical region of protection was obtained using wt and hrN2 fragments labeled at the opposite end (nt 5262) with ^{32}P (data not shown). Using these conditions, no protection was detected in either the wt or hrN2 fragments corresponding to enhancer elements B or E. Footprint analyses also were performed using nuclear extracts from differentiated F9 cells and human HeLa cells (Fig. 4). The same region of protection (nt 5155–5174) again was observed with *Pvu* II fragment 4 from the hrN2 (Fig. 4) and wt (data not shown).

The polyoma nucleotide sequence around the region of the footprint is shown in Fig. 5. A 6-bp inverted repeat is located precisely within the boundaries of the region of nuclease protection. The repeated sequences (5' AGTTGC 3') are separated by 3 bp. A single copy of this sequence is located on the early side of the polyoma origin of DNA replication (nt 63–68, Fig. 1). We tested whether this sequence was capable of binding the factor using the filter binding assay. A fragment spanning this region (nt 5262–162, Fig. 1) was used with
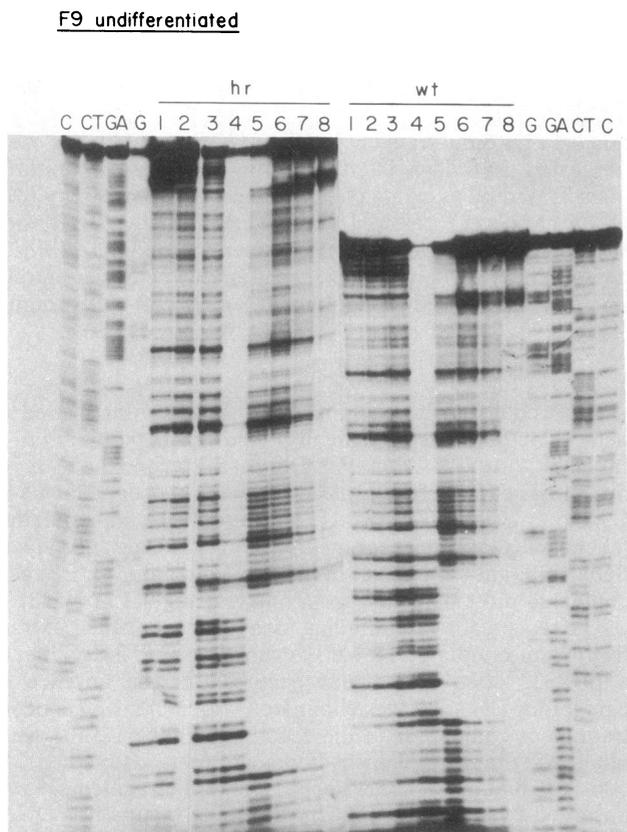
FIG. 3. DNase I footprint analyses of *Pvu* II fragment 4 from wt and hrN2 using nuclear extracts prepared from undifferentiated F9 cells. wt and hrN2 (hr) fragments were ^{32}P-labeled adjacent to the *Pvu* II site at polyoma nt 5128. Lanes 1–4 are ladders obtained when labeled DNAs were treated with DNase I without previous incubation with nuclear extract. Lanes 5–8 are ladders obtained when labeled DNAs were treated with DNase I after incubation with nuclear extract (31 μg of protein). The 250-μl reaction mixtures contained 5.25 μg of competitor DNA and 30,000 cpm of labeled DNAs. Concentrations of DNase I were as follows. Lanes: 1, 0.012 unit/ml; 2, 0.024 unit/ml; 3, 0.048 unit/ml; 4, 0.24 unit/ml; 5, 1.2 unit/ml; 6, 0.48 unit/ml; 7, 0.24 unit/ml; 8, 0.048 unit/ml. Lanes G, GA, CT, and C show homologous sequencing ladders.
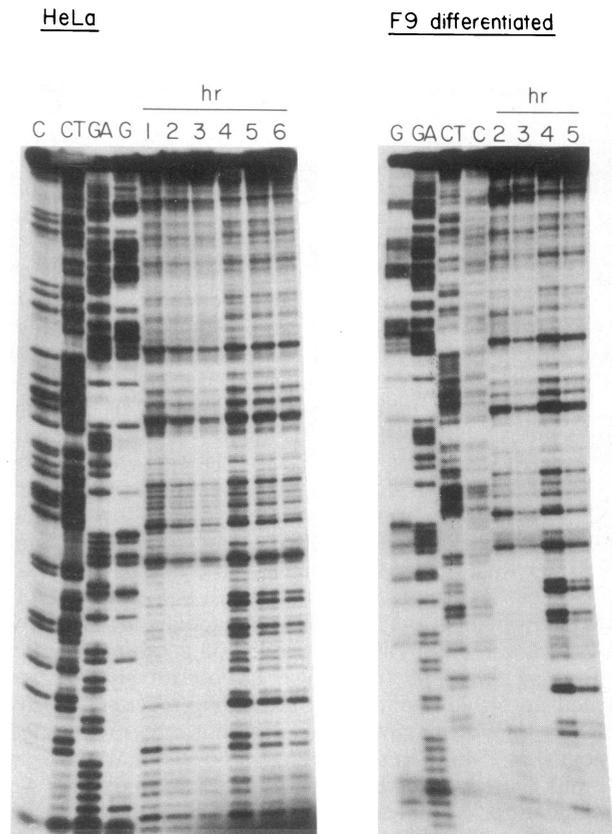
FIG. 4. DNase I footprint analyses of the hrN2 *Pvu* II fragment 4 using nuclear extracts prepared from HeLa cells and differentiated F9 cells. The hrN2 fragment was labeled adjacent to the *Pvu* II site at polyoma nt 5128. For HeLa cell extracts, 250-μl reaction mixtures contained 5.3 μg of competitor DNA, 32.5 μg of protein, and 30,000 cpm of labeled DNA. For differentiated F9 cell extracts, 250-μl reaction mixtures contained 5.3 μg of competitor DNA, 32.8 μg of protein, and 30,000 cpm of labeled DNA. Lanes 1–3 are ladders obtained when labeled DNAs were digested with DNase I after incubation with nuclear extract. Lanes 4–6 are ladders obtained when labeled DNAs were digested with DNase I without prior incubation with nuclear extract. Concentrations of DNase I were as follows. Lanes: 1, 1.2 unit/ml; 2, 0.48 unit/ml; 3, 0.24 unit/ml; 4, 0.048 unit/ml; 5, 0.024 unit/ml; 6, 0.012 unit/ml. Homologous sequencing ladders are shown.

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                                5155                              5174
                          _____

                          pUC | Py              ⟶    ⟵           Py | pUC
                               |                                    |
AAGCTTGGCTGCAGGTCGACGGATCAGTTTTAATTAGTTGCTAGGCAACTGGCCTCGAGGGGGAATTC
└────┘        └────┘         |                                │          └────┘
Hind III       Sal I       n.5150                           n.5178        Eco RI
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FIG. 5. Nucleotide sequence of the region around the EF-C footprint and structure of the pUC-EFC plasmid clone. Polyoma (Py) nt 5150–5178 were cloned into pUC9. The pUC and polyoma sequence boundaries are indicated as are relevant restriction nuclease sites. The region of nuclease protection due to bound EF-C (nt 5155–5174) is indicated by the bar above the sequence. The inverted repeat described in the text (5′ AGTTGC 3′) is indicated by arrows.

nuclear extracts from undifferentiated F9 cells and HeLa cells under a variety of binding conditions. No specific binding to this region was detected in assays containing KCl concentrations from 5 mM to 100 mM and containing protein (extract) concentrations from 1 μg to 25 μg per 50 μl assay (data not shown).

To test whether sequences outside the footprint are required for binding, a plasmid (pUC-EFC) was constructed that contains polyoma nt 5150–5178 inserted in pUC9 (Fig. 5). The polyoma sequences in this plasmid contain the region of nuclease protection (nt 5155–5174) plus only 5 bp upstream and 4 bp downstream from this region. This fragment was excised from the vector using restriction sites that flank the polyoma sequences (Fig. 5, HindIII and EcoRI) and leave 31 bp upstream and 15 bp downstream from the protected region. Specific binding of this fragment was detected using nuclear extracts from undifferentiated F9 cells under conditions that were identical to those established for binding to the entire Pvu II fragment 4 region (data not shown). DNase I footprint analysis demonstrated that an identical region of protection was obtained with this fragment as with Pvu II fragment 4 from wt polyoma and hrN2 (Fig. 6).

DISCUSSION

We have identified a nuclear factor that specifically binds to the polyomavirus enhancer region. This factor is present in nuclear extracts prepared from undifferentiated and differentiated F9 murine embryonal carcinoma cells as well as differentiated murine L and human HeLa cells. The binding activity in each of these extracts is similar, suggesting that expression of this factor is not regulated by the state of cell differentiation. The fact that this factor is found in mouse and human cells is not surprising since the polyoma enhancer region functions in both cell types (31). The region of nuclease protection detected after factor binding spans 20 bp and is located between nt 5155 and 5174. Our results demonstrate that these sequences, and at most 5 bp upstream and 4 bp downstream from this region, contain an intact binding domain. Because of the nature of the assay used in these studies, binding of DNA–protein complexes to nitrocellulose, we suggest that this factor is a protein. Under the conditions used for preparation of nuclear extracts and *in vitro* binding assays, we have not detected other factors that interact with the polyoma Pvu II fragment 4.

The region of nuclease protection conferred by factor binding (nt 5155–5174) corresponds precisely to enhancer element C (nt 5148–5179, Fig. 1) defined by the genetic analyses of Veldman *et al.* (14). This region is required for efficient polyoma DNA replication *in vivo* (7, 14). Enhancer element C in conjunction with element A (the E1A enhancer homology, Fig. 1) represent the predominant enhancer elements in the polyoma enhancer region that are required for viral DNA replication (14). Specific deletion of these two elements (deletion of nt 5101–5165) abolishes replication enhancer function of the entire enhancer region (nt 5021–5262; ref. 14). Enhancer element C also regulates transcription of both the early and late regions of the polyoma genome

(7, 22). We define the factor that interacts with this region as EF-C, enhancer binding factor to polyoma element C.

The boundaries of the EF-C binding domain also correlate with the Ω regulatory region (nt 5157–5181) defined by Maione *et al.* (32). The integrity of this region is maintained in all viable polyoma host range mutants that have been isolated on various undifferentiated murine cell lines. These mutants contain a variety of genetic alterations in the polyoma enhancer region (nt 5021–5262) that include point mutations, deletions, insertions, and rearrangements. It ap-
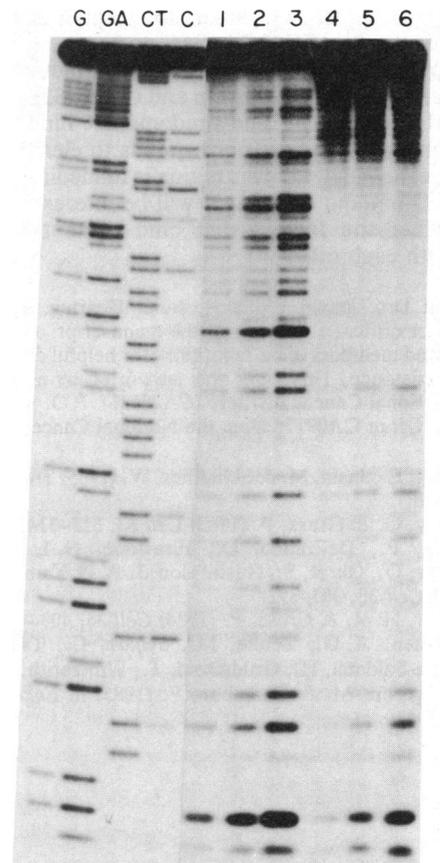
FIG. 6. DNase I footprint analysis of a minimal EF-C binding domain using nuclear extracts prepared from undifferentiated F9 cells. The pUC-EFC clone shown in Fig. 5 was ^{32}P-labeled at the HindIII site, and the HindIII–EcoRI fragment was used for nuclease footprint analysis following a standard binding reaction. The 250-μl reaction mixtures contained 7.5 μg of competitor DNA, 85 μg of protein, and 30,000 cpm of labeled DNA. Lanes 1–3 are ladders obtained when labeled DNAs were digested with DNase I without prior incubation with nuclear extract. Lanes 4–6 are ladders obtained when labeled DNAs were digested with DNase I after incubation with nuclear extract. Concentrations of DNase I were as follows. Lanes: 1, 0.012 unit/ml; 2, 0.024 unit/ml; 3, 0.048 unit/ml; 4, 0.24 unit/ml; 5, 0.48 unit/ml; 6, 1.2 unit/ml. A homologous sequencing ladder is shown. The bar adjacent to the footprint corresponds to the bar above the sequence in Fig. 5.

pears likely that enhancer element C defined by Veldman *et al.* (14) and the Ω regulatory region defined by Maione *et al.* (32) are equivalent and that the function of this region is mediated by EF-C binding.

A 6-bp inverted repeat, separated by 3 bp, is contained symmetrically within the boundaries of the region of nuclease protection conferred by EF-C binding. The EF-C protein, therefore, may bind as a dimer. Two lines of evidence support this possibility. First, a number of DNase I hypersensitive cleavage sites were observed that are located outside the footprint (for example, see Fig. 3). While the intensity of these sites varied from experiment to experiment, the same DNase I hypersensitive cleavage sites were consistently observed and map to nucleotides that are 6, 9, and 11 bp on the 5' side of the upstream repeat and 7, 10, and 12 bp on the 3' side of the downstream repeat. Thus, hypersensitive sites induced by EF-C binding symmetrically flank the inverted repeat sequence. Second, a single copy of the repeated sequence located on the early side of the polyoma replication origin did not detectably bind EF-C. Thus we speculate that EF-C binding is stabilized by dimerization.

Several other groups have defined nuclear factors that interact with the polyoma *Pvu* II fragment 4. The factor described by Fujimura (33) binds to a region that precisely corresponds to the EF-C binding domain and these two factors are undoubtedly the same. Nuclear factors identified by Piette *et al.* (34) and Bohnlein and Gruss (35) also bind to the *Pvu* II fragment 4. The predominant binding activity observed by these authors corresponds to element B of the enhancer region (Fig. 1). This region is immediately adjacent to the EF-C binding domain. It will be interesting to determine whether the factors that bind these two elements interact with each other.

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