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Point mutation in the polyomavirus enhancer alters local DNA conformation

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ABSTRACT

A point mutation in the enhancer of polyomavirus host range mutant, PyEC F441, permits productive infection of the murine embryonal carcinoma cell line, F9. This mutation at nucleotide position 5258 introduces a local conformational change in naked viral DNA. The effect of all four possible nucleotide sequences at position 5258 on local DNA conformation was analyzed by gel electrophoresis of fragments produced by ligation of synthetic oligonucleotides having these sequences. The results indicated that both the wild-type and the F441 sequences introduced local structural polymorphism that can lead to DNA bending. The wild-type sequence had a greater effect on DNA curvature than the F441 sequence. The two other sequences at nucleotide 5258 did not appear to introduce detectable amounts of DNA curvature.

INTRODUCTION

Murine embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, are refractory to infection by wild-type polyoma virus [1]. Differentiation of EC cells leads to cell types that are permissive for polyoma infection [1-4]. A number of host range mutants of polyoma virus that do infect EC cells, as well as other relatively undifferentiated mouse cell lines, have been isolated and characterized [5-10]. All of these mutants have DNA sequence alterations within the region of the polyoma genome defined as the enhancer [11]. Interestingly, polyoma mutants isolated on one EC cell line do not necessarily infect other EC cell lines. Several mutants isolated on PCC4 EC cells do not infect F9 EC cells; conversely, mutants isolated on F9 cells do not infect PCC4 cells very efficiently. This difference in host range properties is reflected in the regions of the polyoma enhancer that are altered in these two groups of mutants. All mutants infecting F9 cells have a specific A:T to G:C transition at nucleotide 5258 [6,7,12]. Marker rescue experiments have shown that this point mutation, found in the enhancer of the PyEC mutant PyF441, is sufficient for infection of F9 cells [6]. Tandem duplication of sequences encompassing this mutation results in more efficient infection of F9 cells [6]. Mutants

infecting PCC4 cells never have this point mutation at nucleotide 5258. In fact, these mutants have deletions of sequences adjacent to, and sometimes, including nucleotide 5258 [5]. Thus, the host range of polyoma virus may depend upon different domains within the enhancer which could have different cell specificities. In light of the apparently unique specificity of the F441 mutation for infection of F9 cells, synthetic oligonucleotides corresponding to the sequences of all four possible base pairs at this site were analyzed. The results indicate that the F441 mutation affects DNA conformation in a manner that is clearly distinguishable from the three other sequences.

MATERIALS AND METHODS

DNaseI and Copper-Phenanthroline Cleavage of DNA

The Msp I fragment 3 of wild-type, PyF111 and PyF441 DNAs were labeled with ^{32}P at either the 5′ or 3′ ends, digested with Bcl I, and the smaller end-labeled fragment was isolated [13]. The purified probes were labeled at the Msp I site at nucleotide 25, with the E-strand being 3′-end-labeled or the L-strand being 5′-end-labeled. The asymmetrically end-labeled fragments were digested with DNase I, then analyzed by electrophoresis in a 6% acrylamide sequencing gel as described previously [13]. These labeled fragments were also subjected to cleavage by copper-phenanthroline complex exactly as described by Cartwright and Elgin [14] and cleavage products were analyzed in the sequencing gels.

Oligonucleotides

The oligomers used in the present studies are shown in Table 1. These oligomers were synthesized to correspond to polyoma viral DNA sequences surrounding the PyF441 point mutation at nucleotide 5258. Complementary strands were synthesized to produce 2 base pair overhangs at the 5′ ends. Complementary strands were annealed by heating briefly at 55°C, then cooling slowly to room temperature. Duplexes were purified by electrophoresis in acrylamide gels. After staining with ethidium bromide, duplex bands were electroeluted onto DEAE-nitrocellulose membranes (NA45, Schleicher and Schuell). The membranes were washed and oligomers were eluted with 1 M NaCl according to the manufacturer's instructions. The oligomers were further purified by retention on a NACS Prepac column (Bethesda Research Laboratories) in 0.1 M NaCl, followed by elution with 1 M NaCl. The purified duplex oligomers were concentrated by precipitation with 67% ethanol in the presence of 6 μg/ml oyster glycogen.

Table I. Sequences of Oligomers

NAME	SEQUENCE (5′ - 3′)
	*
WT(10)	GGCCTAGAAT
F441(10)	GGCCTGGAAT
A/C(10)	GGCCTCGAAT
A/T(10)	GGCCTTGAAT
WT(12)	AGGCCTAGAATG
F441(12)	AGGCCTGGAATG
	*

Only the sequence corresponding to the E-strand of polyoma DNA is shown. The 5′-terminal A of the 12-mers corresponds to polyoma nucleotide number 5252 [37]. The asterisk (*) indicates nucleotide 5258, site of the F441 point mutation.

Ligation Ladder Analysis

Approximately 500 ng of each duplex was end-labeled by treatment with 1 μCi of γ-^{32}P-ATP (New England Nuclear, ca. 300 Ci/mmole) and 6 units of T4 polynucleotide kinase (Pharmacia Molecular Biologicals, 10 units/μl) for 15 min at 37°C in 10 μl of 70 mM Tris-Cl (pH 7.4), 10 mM $MgCl_2$, 5 mM dithiothreitol. Then, after addition of unlabeled ATP to a final concentration of 2.6 mM and 5 more units of polynucleotide kinase, the reaction was incubated for 1 hr at 37°C. Reactions were cooled on ice and, after addition of 1 unit of T4 DNA ligase (IBI, 1 unit/μl), were incubated at 4°C for times appropriate to give useful fragment ladders. Ligated fragments were analyzed by electrophoresis in polyacrylamide gels run either at room temperature or in a cold room at 4 volts/cm using TBE [15] buffer. HinfI fragments of wild-type polyoma DNA, end labeled with polynucleotide kinase as above were used as size markers. In addition, a 10-mer ladder, M(10), obtained by blunt-end ligation of kinased SalI/SmaI adapter (dTCGACCCGGG, New England Biolabs), which behaves normally compared to the HinfI marker fragments, was run in these gels. After electrophoresis, gels were dried and exposed to X-ray film using intensifying screens at -90°C. Mobilities of individual band were measured and the apparent size of each fragment was determined relative to the M(10) fragment ladder.

RESULTS AND DISCUSSION

Previous work from this laboratory used DNase I [16] and Exo III [17] footprinting techniques to identify a factor or factors from F9 EC cells

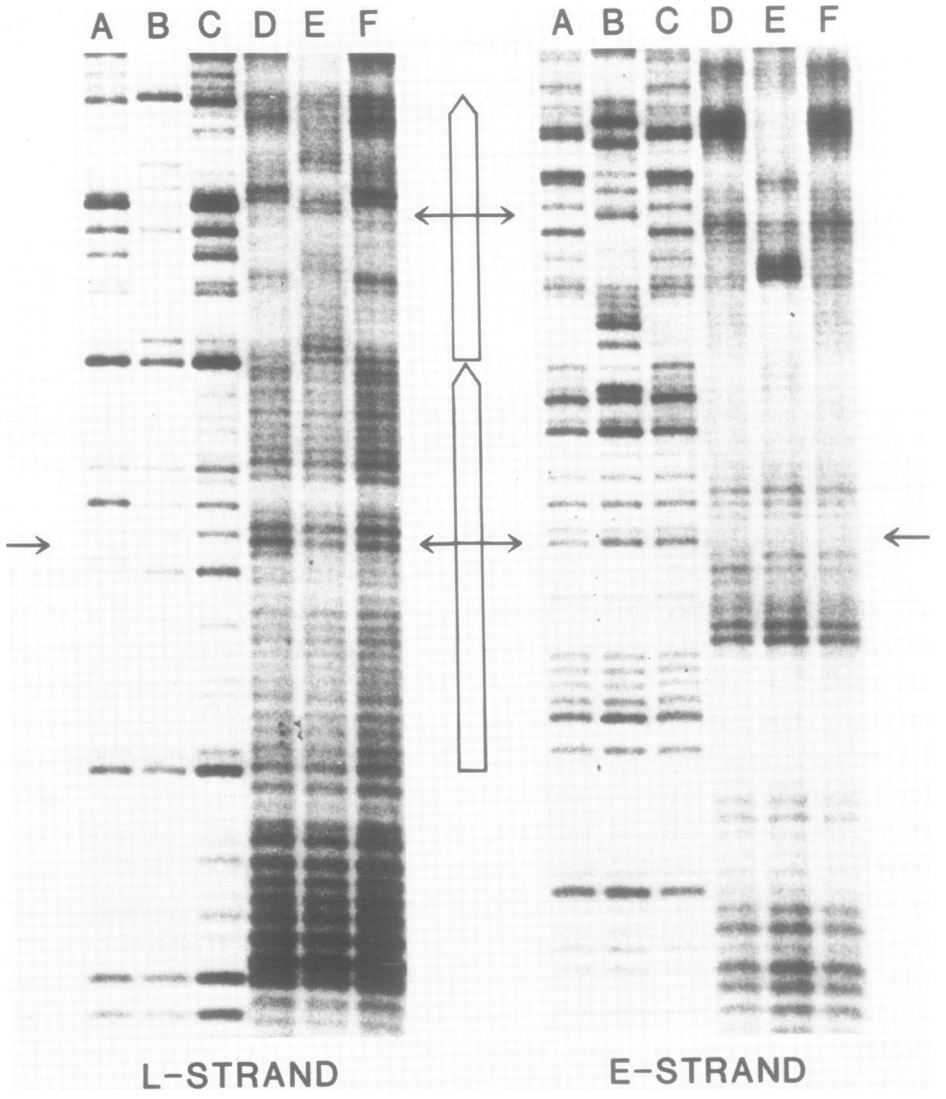

Figure 1. DNase I and copper-phenanthroline cleavage of wild-type and PyEC DNAs. DNA fragments, labeled in either the E-strand or the L-strand at the Msp I site at nucleotide 24, were digested with DNase I (A-C) or copper-phenanthroline (D-F) and analyzed in a 6% acrylamide, 8 M urea sequencing gel. DNAs were from wild-type polyoma (A,D), PyF111 (B,E) and PyF441 (C,F). The arrows designate nucleotide 5258, which is the site of the point mutation in PyF441 DNA. The nucleotide sequence of PyF111 is identical to that of PyF441 except for a tandem duplication of 31 nucleotides symmetrically surrounding the point mutation with both copies of the duplication containing the PyF441 point mutation. The F111 tandem duplication is represented by the pointed bars.

binding specifically to a small region of the polyoma enhancer [13]. During those experiments, a difference between wild-type and PyF441 was noticed in the DNase I and Exo III digestion patterns on naked, linearized viral DNAs. This difference was localized to the region around the site of the PyF441 point mutation. Figure 1 shows the nicking pattern of DNase I on both strands within the enhancers of wild-type and two PyEC mutant DNAs. The difference in DNase I nicking between wild-type and mutant DNAs was most obvious on the L-strand around the site of the PyF441 point mutation at nucleotide 5258 (indicated by the arrows in Fig. 1). Corresponding differences in relative band intensities localized around the site of the point mutation were also observed on the E-strand. The sensitivity of sites to DNase I cleavage is sequence-dependent as shown by the repeat of the local cleavage pattern for PyF111 DNA, which is identical to PyF441 DNA except for a tandem duplication of 31 nucleotides symmetrically surrounding the site of the PyF441 point mutation [6].

To rule out any peculiarity in cleavage by DNase I, the same end-labeled DNAs were analyzed by electrophoresis after chemical degradation using copper-phenanthroline [14]. While not as striking as DNase I cleavage patterns, slight differences can be detected in the relative intensities of bands around the site of the point mutation between wild-type and mutant DNAs. Thus, this conformational difference between wild-type and mutant DNAs is suggested from results with both enzymatic and chemical probes. This conformational feature is detected on linear DNA so that its presence does not require torsional stress. Whether the conformational feature is affected by energy of DNA supercoiling is not known.

To characterize the nature of the conformation of wild-type and mutant DNAs, synthetic oligomers of these sequences were made and, after ligation, analyzed by polyacrylamide gel electrophoresis. It has been shown [18,19] that sequences which induce DNA bending, when duplicated in phase with the DNA helix by ligation of 10 or 11-mers, result in fragments that migrate anomalously slowly in polyacrylamide gels. This anomalous electrophoretic behavior is reduced significantly when such sequences are duplicated out of phase with the helix by self-ligation of 12 or 15-mers [18,19].

A typical electrophoretic pattern is shown in Figure 2. When compared to the ladder of fragments (lanes M(10)) that migrate normally relative to Hinf I restriction fragments of polyoma DNA (lane H), both wild-type (lane WT(10)) and PyF441 (F441(10)) ladders resulting from ligation of 10-mers are more retarded in the gel. This anomalous electrophoretic behavior is

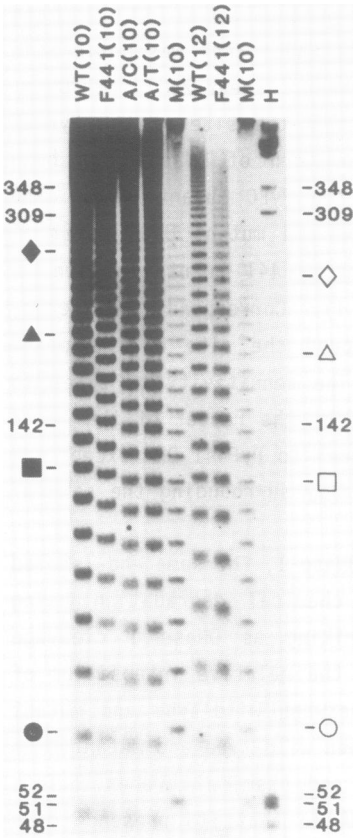

Figure 2. Electrophoretic analysis of ligated oligonucleotides. The oligonucleotides shown in Table 1 were ligated and analyzed in an 8% acrylamide (acrylamide:bis = 20:1) gel run at room temperature. The lanes marked M(10) indicate the ladder of fragments resulting from blunt end ligation of commercial Sal I/Sma I adapter. These fragments migrate normally relative to end-labeled Hinf I fragments of wild-type polyoma DNA (lane H). The sizes, in base pairs, of these Hinf I fragments are indicated. The positions of bands in the WT(10) and M(10) ladders corresponding to 60, 120, 180 and 240 bp are shown:

Base Pairs:	60	120	180	240
WT(10):	●	■	▲	◆
M(10):	○	□	△	◇

dependent upon these sequences being repeated in phase with the double helix, because ligation of wild-type and PyF441 12-mers (WT(12) and F441(12), respectively) results in more normal ladders. Wild-type ladders consistently exhibited greater electrophoretic anomaly than the corresponding PyF441 ladder, indicating that, although both sequences affect DNA curvature, the wild-type sequence is more efficient than the PyF441 sequence for bending DNA.

The behavior of wild-type and PyF441 ladders at 4°C and at room temperature is represented graphically in Figure 3A,B. The anomalous mobilities of WT(10) and PyF441(10) fragments are accentuated at the lower temperature. It has been shown that the electrophoretic mobility of kinetoplast DNA, the classic example of a naturally bent DNA [20], is more abnormal at lower

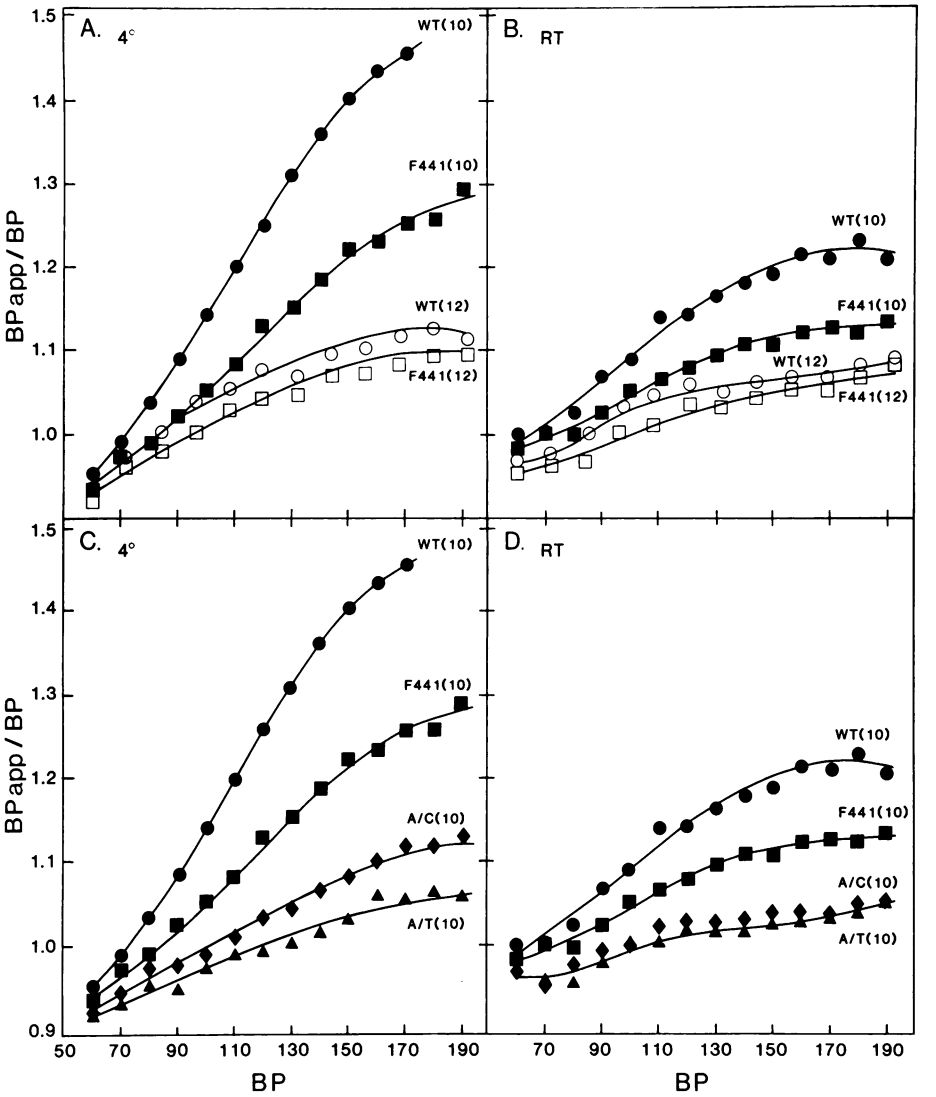

Figure 3. Temperature-dependence of electrophoretic mobilities of ligated oligomers. Ligated oligonucleotides, as in Figure 2, were analyzed by electrophoresis in a cold room (4°) or at room temperature (RT) in 12% acrylamide (acrylamide:bis = 30:0.8) gels. Apparent sizes (BP_{app}) of fragments were determined with respect to the M(10) ladder which behaves normally compared to Hinf I fragments of wild-type polyoma DNA (see Fig. 2). The ratio of the apparent size to the actual size of fragments (BP_{app}/BP) is plotted against the actual fragment size (BP).

temperature [21]. Accentuation of anomalous mobility by reduced temperature provides one criterion for DNA bending. This effect of temperature coupled with the fact that anomalous electrophoretic behavior due to conformational states invoking local helix instability or flexibility would not be dependent upon helix phasing [18], strongly support the idea that the effect observed for these polyoma enhancer sequences is due to local helix deformation resulting in DNA bending.

Because both the wild-type and PyF441 10-mer ladders showed anomalous electrophoretic mobilities, the behavior of sequences having the two other possible base changes at nucleotide 5258 was examined. Ligation ladders of 10-mers having A to C (A/C(10)) or A to T (A/T(10)) transversions at nucleotide 5258 were compared to those of wild type and PyF441 10-mers. As can be seen in Figure 3C,D, A/C(10) and A/T(10) ladders behaved more normally than the F441(10) ladder. Comparison with the data in Figure 3A,B indicates that the A/C(10) and A/T(10) ladders are similar to the wild-type and PyF441 12-mer ladders. Thus, of the four possible nucleotide pairs at position 5258, the two found in wild-type and F441 affect DNA conformation to a greater degree than the two other possibilities. It should be noted that, while differences in mobilities among the experimental 10-mer ladders were always consistent among themselves, all experimental 10-mer fragments smaller than approximately 70 base pairs migrated faster than the corresponding fragments of the M(10) ladder. Retardation of WT(10) and F441(10) ladders relative to the marker ladder became apparent for fragments larger than approximately 80 base pairs.

The four possible sequences at nucleotide 5258 fall into three groups with respect to the magnitudes of their effect on local DNA conformation, with the wild-type sequence having the greatest effect, the two transversions having virtually no effect, and the F441 sequence having an intermediate effect. Plasmid constructs and complete polyomavirus genomes having these four possible sequences have been constructed and analyzed [R. W. Tseng, T. Williams, and F. K. Fujimura, manuscript submitted]. Although all four viral constructs infect mouse 3T6 cells efficiently, only F441 infects F9 cells. It may be relevant that the effects of these four sequences in plasmid constructs with regard to enhancement of expression of chloramphenicol acetyltransferase by transient transfection assays of F9 and 3T6 cells also fall into three categories, with F441 being more active than wild-type and the two transversion mutants apparently inhibiting enhancer activity with regard to the wild-type sequence. It may be significant that the three

groups inferred from the conformational studies described here correlate with the three functional groups observed in transfection assays.

It should be noted that the effects of these polyoma DNA sequences on DNA curvature are quite small compared to effects observed for sequences that strongly bend DNA [18,19,22]. However, the biological relevance of DNA conformation to function is not well defined, so there is no reason to rule out the possibility that biological regulation may depend on subtle conformational features of DNA. In fact, it has been argued [23] that such conformational features in DNA, rather than major structural variations, may be the important determinant of biological specificity. Perhaps relevant are recent observations [24,25] suggesting that enhancer activity, at least during a transient transfection assay, does not depend upon DNA superhelicity or torsional stress due to supercoiling.

Several observations do suggest the possible involvement of DNA bending in biological function. Bossi and Smith [26] found that a 3 base pair deletion in a region upstream of the promoter site of a tRNA operon in Salmonella, not only eliminated the anomalously slow electrophoretic mobilities of restriction fragments containing this region, but also resulted in a decrease in transcriptional efficiency of this promoter in vitro, suggesting a role for DNA bending in transcriptional initiation at this promoter. Bent DNA has also been observed in the replication origins of several DNAs including those of simian virus 40 [27], phage lambda [28], and an autonomously replicating sequence of yeast [29]. Furthermore, several regulatory proteins, upon binding to DNA, induce DNA curvature. Among these proteins are the cyclic AMP receptor (CAP) protein [30], replication initiator proteins of plasmids R6K [31] and pT181 [32], and the repressor [33] and the O-protein [34] of phage lambda. The relevance of DNA bending with regard to the mechanism of action of these proteins is not known, but it has been suggested [33,35,36] that DNA bending may be important for aligning protein-protein interactions in protein-DNA complexes.

Present results indicate that, although DNA bending or bendability may be important for polyoma enhancer function, the host range of the virus with regard to infection of F9 cells cannot be explained by the simplest model invoking DNA bending: that the degree of bending determines the efficiency (or inefficiency) of infection of F9 cells. Instead, the unique conformation due to the F441 sequence, rather than DNA bending per se, may be the relevant parameter for enhancer activation. It is also possible that a combination of effects due to sequence and conformation is involved in activation. Because

the effects of the sequences examined here on DNA conformation are very small, the possibility that many other sequences may also have similar effects on conformation cannot be ruled out. Therefore, it would be premature to argue strongly for the unique role of DNA conformation in regulation of polyoma enhancer activity in F9 cells. What is clear is that the F441 point mutation has significant and specific effects on both transcription and replication of the polyoma genome in EC cells. Recently, nuclear factor(s) from F9 and some other cell lines has been shown to bind specifically to the F441, but not the wild-type, enhancer sequence [38,39]. Work from this laboratory [R.W. Tseng, T. Williams, and F.K. Fujimura, manuscript submitted] has shown that purified CCAAT-box transcription factor from HeLa cells, which is identical to nuclear factor 1 [40], can distinguish the F441 sequence from the three other possible sequences at nucleotide 5258. Thus, the F441 mutation could create a specific binding site for a positive-acting regulatory factor. It is possible that binding of this putative regulatory factor will depend, in part, on specific conformational alteration in the polyomavirus enhancer due to the F441 point mutation.

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