Repression of Polyoma Virus DNA Replication by 5’-Flanking Region of Mouse DNA Polymerase β Gene Containing Transcriptional Silencer Elements*

Masamitsu Yamaguchi and Akio Matsukage
From the Laboratory of Cell Biology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

(Received for publication, January 13, 1989)

Dual cis-acting silencer elements are located upstream of the mouse DNA polymerase β gene (Yamaguchi, M., Hayashi, Y., and Matsukage, A. (1989) J. Biochem. (Tokyo) 105, 79–83). In order to examine possible involvement of transcriptional silencer elements in the regulation of DNA replication, we have utilized a transient replication system of the plasmid DNA carrying replication origin of polyoma virus DNA in mouse MOP8 cells, which is constitutively producing polyoma virus large T-antigen. The polyoma virus origin of DNA replication is composed of three cis-acting genetic elements called α, β, and core, in which α and β elements correspond to enhancer domains. When the 5’-flanking regions of the DNA polymerase β gene containing silencer elements were placed at the late gene border of α element, they effectively repressed the DNA replication. However, when placed at the early gene border of core element, it only marginally repressed the DNA replication. These results suggest that the silencer elements at cis position repress polyoma virus DNA replication by impeding the enhancer function that activates the DNA replication.

The functional origin (ori) of DNA replication in eukaryotic genome frequently contains transcriptional regulatory elements as its integral components (1, 2). In simian virus (SV) 40, the ori-core is flanked by two auxiliary sequences that can enhance DNA replication severalfold (3, 4). One of these auxiliary sequences resides within the promoter region of early gene (3, 4), and SV40 enhancer can compensate the effect of this auxiliary element when it is juxtaposed to the AT-rich side of SV40 ori-core (3–6). At least one of the enhancer elements is essential to activate ori-core sequence in polyoma virus DNA replication (7, 8). Furthermore, cellular proteins called NF-I and NF-III, both of which are required for the replication of adenovirus DNA, can bind specifically to promoter and/or enhancer sequences of certain cellular genes (9, 10). Involvement of c-myc protein in replication and transcription of its own gene was also suggested (11, 12). Thus, it is likely that complex circuits between replication and transcription actually function in eukaryotic cells.

Recently, we have identified the dual cis-acting negative regulatory elements in the upstream region of mouse DNA polymerase β gene. These elements act on the promoters of heterologous genes, relatively independently to the distance from the promoter and to the orientation relative to the promoter (13). These properties are very similar to those of the so-called transcriptional silencer element (14). Since one of the sub-elements of yeast silencer that is involved in repression of mating type loci is reported to function as an autonomous replication sequence (15), it is probable that a silencer element regulates not only transcription but also DNA replication in higher eukaryote.

In the present study, we have utilized the transient replication system of polyoma virus DNA in MOP8 cells, which has been extensively characterized (16), to examine the effect of DNA polymerase β gene silencer elements on the DNA replication. The results indicate that silencer elements-containing sequence can efficiently repress polyoma virus DNA replication presumably by impeding the reactions taking place in the late gene side of the replication origin.

MATERIALS AND METHODS

Plasmid Constructions—A 1535-base pair (bp)1 DNA fragment containing DNA polymerase β gene silencer elements (nucleotide position −1860 to −327, where +1 denotes the position of 5’-most proximal transcription initiation site) (see Fig. 1) was isolated from one of the 3’-end deletion derivatives of pUC19-MG201 (17), blunt-ended, and inserted into the blunt-ended unique XhoI site of the plasmid pPyLCAT (18). The obtained plasmids containing the silencer elements in both normal and reverse orientations were designated as pPyLoNRE(NCAT) and pPyLoNRE(RCAT), respectively (see Fig. 2). The same silencer fragment attached with BamHI linkers at both ends was inserted into a unique BamHI site of the plasmid pPyECAT (19) in both normal and reverse orientations to create the plasmids pPyEβNRE(NCAT) and pPyEβNRE(RCAT), respectively. The same fragment attached with BamHI linkers at both ends was inserted into a unique BamHI site of the plasmid pUC19NRE. These plasmids were propagated in Escherichia coli HB101 and isolated by standard procedures (20). Isolated plasmid DNA was further purified through 2 cycles of ethidium bromide/CsCl density gradient centrifugation (20). The validity of the obtained recombinant plasmids was examined by restriction endonuclease mapping (21) and dyeoxy sequencing method (22, 23) using a synthetic 17-mer primer (5’-TTTATGTTAAGTTCAGACA-3’) that hybridized to nucleotide position −456 to −440 of DNA polymerase β gene.

DNA Transfection—MOPS cells (16) were cultured in Dulbecco’s modified minimal essential medium supplemented with 10% (v/v) fetal calf serum. The DEAE-dextran method with chloroquine treatment (24) was used for DNA transfection into cells at 20 h after seeding 3.0 × 105 cells per 100-mm dish. Each transfection contained 5 μg of plasmid DNA per dish. The amount of DNA used for transfection (between 1 and 10 μg per dish) did not change the replicative capacity of the recombinant plasmids. Cells were harvested 72 h after transfection and processed for DNA replication assay. Transfections were done multiple times to avoid errors caused by

* This research was supported in part by a Grant-in-Aid for Cancer Research from the Japanese Ministry of Education, Science, and Culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 052-762-6111; Fax: 052-765-5233.

1 The abbreviations used are: bp, base pair(s); kb, kilobase pair(s).
Repression of DNA Replication by Silencer

FIG. 1. Organization of transcriptional regulatory elements for mouse DNA polymerase \( \beta \) gene. Nucleotide +1 denotes the 5' most proximal major cap site, and the residues preceding it are shown by negative numbers. The regions of transcriptional silencer elements 1 and 2 are indicated by shaded blocks (13). Exons 1 and II are indicated by solid boxes, and the translation initiation codon is indicated as ATG (17). Two major cap sites for DNA polymerase \( \beta \) mRNA are indicated by vertical lines with arrows (17). The involvement of Spl-like, USF-, and/or ATF-like mouse transcription factors in the promoter function was suggested by Yamaguchi et al. (26). The indicated region between -1860 and -327 was cloned into pPyECAT or pPyLCAT in both normal and reverse orientations (see Fig. 2).

Fluctuation in transfection efficiency.

DNA Replication Assay—72 h after DNA transfection, low molecular weight plasmid DNA was isolated from MOP8 cells by the Hirt (25) extraction method. After the sedimentation of high molecular weight DNA, 1.5 ml of the cleared lysate was mixed with 3 ml of TE buffer (10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA) and extracted once with buffer-saturated phenol and once with chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated by adding 2.5 volumes of ethanol. Precipitates were collected by centrifugation and suspended in 50 \( \mu l \) of TE buffer. A 15-\( \mu l \) portion of the DNA sample was digested with BamHI and DpIII for the plasmid pPyECAT and its derivatives and with EcoRI and BpIII for the plasmid pPyLCAT and its derivatives. The digested plasmid DNAs were subjected to electrophoresis through 1.0% agarose gels, and DNA fragments were transferred to Genescreen Plus filters (Du Pont-New England Nuclear). The filters were hybridized with \( ^{32}P \)-labeled pPyLmNRE(N)CAT DNA (5 \( \times 10^8 \) cps/\( \mu g \)). After washing, the filters were dried and autoradiographed for 3 to 16 h with Kodak XAR-5 x-ray film and Du Pont Lightning Plus intensifying screen. The intensities of radioactive bands were quantified by densitometry. Each recombinant plasmid DNA was assayed in duplicate on three separate occasions, and the duplicate samples were processed separately.

RESULTS

Effect of DNA Polymerase \( \beta \) Gene Silencer Elements on the Polyoma Virus DNA Replication—By analyzing a set of 5'-end deletion mutants in the 5' flanking region of the mouse DNA polymerase \( \beta \) gene, we have identified the two tandemly aligned, negatively acting regions for the gene expression that functioned in mouse NIH/3T3 cells (13). One region resides within the 280-bp region between -1860 and -1580 and the other regions within the 370-bp region between -828 and -456 (13). These regions have properties of so-called transcriptional silencer element (13). (i) They repressed the
function of the promoter-enhancers of heterologous genes such as SV40 early gene, Rous sarcoma virus long terminal repeat, and polyoma virus late gene, in addition to the promoter of its own gene. (ii) They repressed the gene expression relatively independently of the orientation to the promoter-enhancer and the distance from the promoter-enhancer. A DNA fragment containing DNA polymerase β gene silencer elements (nucleotide position −1860 to −327) was inserted in both normal and reverse orientations into a unique BamHI site of the plasmid pPyECAT. The site resides immediately upstream of the late gene border of the polyoma enhancer (Fig. 2). The same fragment was also inserted into a unique XhoI site of the plasmid pPyLCAT in both normal and reverse orientations. This site resides close to the large T-antigen binding site C (Fig. 2). Constructs of these four plasmids as well as the polyoma virus control region for replication and transcription are illustrated in Fig. 2. In obtained recombinant plasmids, the functional origin of replication (α, β, and core elements) is kept intact, allowing the replication of plasmid DNAs in mouse cells when the polyoma T-antigen is supplied in trans. These plasmids were transfected into MOP8 cells (16), which originated from NIH/3T3 cells and constitutively express polyoma T-antigen from an integrated ori polynoma genome, and their replicative capacities were measured by the conversion of DNA that was sensitive to cleavage with DpnI restriction endonuclease into DNA that was resistant to the nuclease (27). This MOP8 cell system has been proven to support the transient episomal replication of the plasmid DNA carrying polyoma ori (16, 35). Low molecular weight DNA recovered from MOP8 cells were first cut with EcoRI or BamHI to convert all forms of monomeric DNA into two DNA fragments, one of which was derived from the original vector DNA region. All used plasmids containing multiple DpnI sites that were methylated after growth in a dam + E. coli strain and rendered sensitive to cleavage by DpnI. Mammalian cells do not contain the dam-methylase. Therefore, when the plasmid DNA is replicated into MOP8 cells, it turns into a hemimethylated form after 1 cycle of replication and an unmethylated form after 2 or more cycles of replication. Both hemimethylated and unmethylated forms are insensitive to DpnI. Therefore, the amount of DpnI-resistant DNA represents the extent of replication.

The results with the plasmid pPyECAT and its derivatives are shown in Fig. 3. The plasmid pOLCAT which did not carry polyoma ori did not replicate at all (Fig. 3A, lane g), while the plasmid pPyECAT carrying polyoma ori replicated efficiently (Fig. 3A, lanes a and b), indicating that the DNA replication is dependent on the polyoma ori sequence. This fact also indicates that the DpnI-resistant bands are not produced by repair type DNA synthesis. These data confirm the previous reports showing the polyoma ori dependency of
the transfected plasmid DNA replication in MOP8 cells (16, 35). The previous reports also show the polyoma large T-antigen dependency of the plasmids carrying polyoma ori in MOP8 cells (16, 35). The derivatives carrying DNA polymerase β gene silencer elements replicated less efficiently than the pPyECAT and resulted in the reduction of the amount of DpnI-resistant fragments by about 70% (Fig. 3A, lanes c to f). As shown in Fig. 3B, the amount of total plasmid DNA recovered from the cell without DpnI-digestion is almost same as DpnI-resistant DNA, indicating that the plasmid DNAs not replicated are lost from cells within 72 h after transfection.

The results with the transfected pPyLCAT and its derivatives are shown in Fig. 4. The 4.5-kb fragment containing silencer elements migrated more slowly than the 3.0-kb fragment derived from original vector DNA. The amounts of 2.1-kb fragments derived from the vector region of the plasmid DNA that replicated in MOP8 cells were compared. Average values (±S.D.) from these results and those from two other independent experiments are given at the top of each lane. In contrast to the results shown in Fig. 3, DNA polymerase β gene silencer elements affected only marginally the DNA replicative capacity of the plasmid pPyLCAT DNA in cis and not at all in trans (Fig. 4). Almost no DpnI-digested fragments were detected in the low molecular weight region of the gel, indicating the quick loss of unreplicated DNA from MOP8 cells, such as the case with the transfected pPyECAT and its derivatives.

**DISCUSSION**

The 5′-flanking region of the DNA polymerase β gene containing the transcriptional silencer elements efficiently repressed the polyoma virus DNA replication when it was placed in the late side border of the enhancer region (Fig. 3). In polyoma virus, either the α or β element must be juxtaposed next to core element to form a functional origin of DNA replication (1, 8, 19). These α and β elements correspond to enhancer elements A and B + C, respectively (Fig. 2), and they function redundantly to each other to activate transcription (1, 8, 19). The α and β elements activate replication independently of their orientation to the core element. However, unlike enhancer elements, they cannot activate the DNA replication when placed a long distance from the core element (19). Thus, even if the initial binding of the trans-acting regulatory factor(s) to the enhancer element(s) is common for activation of both transcription and DNA replication, the subsequent pathways used for these two processes might be different (19). It is suggested that α and β elements facilitate binding of T-antigen to core element either directly through interaction with the initiation complex for DNA replication containing T-antigen or indirectly by exposing the ori sequence that is normally present in inactive chromatin structures open for DNA replication enzymes (28). Since DNA polymerase β gene silencer elements can dominate a number of enhancers such as SV40, Rous sarcoma virus, and polyoma virus to repress transcription, it is probable that the silencer elements repress DNA replication by impeding the activation of the core element by α and β elements.

Two of three sub-elements of yeast MAT locus silencer cooperate to exhibit centromere-like segregation activity, suggesting that silencer function involves an attachment of the DNA to the nuclear membrane or lamina (15, 29). Therefore, it might be possible that the DNA polymerase β gene silencer elements repress DNA replication by tagging the ori-containing plasmid DNA to the special sub-nuclear compartment where the replication enzymes are not available.

It is not known why the DNA polymerase β gene silencer elements can only marginally repress DNA replication when placed in the early gene side of the core element. One possible explanation is that tight binding of T-antigen to high affinity sites A, B, and C (Fig. 2) might block the effect of the silencer sequence. Interestingly, α and β elements cannot activate core element when they were juxtaposed to the early side of core element (30). Asymmetry of ori function also showed that the synthesis of the first nascent DNA chain takes place exclusively on the early mRNA template strand of ori and progresses toward the direction of the early genes (31, 32). Thus, some unknown mechanisms might exist to prevent the action of the silencer elements when they are placed in the early side of the ori.

**Acknowledgments**—We are indebted to Drs. John Hassell (McGill University) and Melvin DePamphilis (Roche Institute of Molecular Biology) for providing MOP8 cells and polyoma ori plasmids.

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Repression of DNA Replication by Silencer

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