cis-Acting Sequences That Control the Level of Viral DNA Synthesis in the Polyomavirus Late Region

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A deletion in the polyomavirus late region results in a drastic reduction of viral replication, as shown after transfection of viral DNA into 3T6 cells. This mutation is *cis* acting, since cotransfection with wild-type DNA did not restore the normal phenotype. Viral DNA synthesis returned to normal levels only after reintroduction of the authentic sequences in either orientation. The data presented here suggest that these sequences are involved in the binding of a factor(s) that controls the level of viral replication.

Polyomavirus (Py) growth regulation has been intensively studied during recent years. However, these studies have focused mainly on the early coding region and the noncoding regulatory sequences of the viral genome (10; M.-H. Krysthe and M. Yaniv, *Crit. Rev. Biochem.*, in press). On the other hand, the mere codification of structural capsid proteins has been ascribed to the late coding region of the Py genome (for a review, see reference 17).

We have performed *in vitro* mutagenesis on several sites of the viral late region and have produced a series of mutants with different phenotypes (G. Risuleo and G. Magnusson, manuscript in preparation). In this paper, we report on a mutant with a deletion of a specific sequence in the viral late region that results in a drastic reduction of the level of viral DNA synthesis.

Deletion mutant construction and characterization. In order to construct the mutant, the Py wild-type (wt) genome, cloned in the *Bam*HI site of pBR322 (13), was linearized by partial digestion with *Hind*II. A synthetic *Bgl*II linker was ligated at either *Hind*II restriction site of the linear products. Ligated circular molecules were used for transformation of HB101 cells. DNA was prepared from transformant colonies, and those containing a recombinant plasmid with the *Bgl*II site at nucleotide position 3466 were identified by double digestion with *Bgl*II and *Bam*HI. After linearization with *Bgl*II restriction endonuclease, the recombinant plasmid was subjected to *Bal* 31 nuclease digestion at a final concentration of 12.5 U/ml. The products of this digestion were recircularized, after ligation of a *Bgl*II linker to their termini, and transformed into HB101. The extent of four different deletions was assessed by DNA sequencing (16). One of these viral mutants, in which nucleotide 4382 is fused to nucleotide 3216 via the *Bgl*II linker (Fig. 1), was used throughout this work and will be referred to as Py-*d*/B3. (The methods used are those reported by Maniatis et al. [15].)

The Py mutant genome was excised from the vector sequences and ligated at a low DNA concentration (5 µg/ml). Viral DNA replication activity was measured after transfection of semiconfluent 3T6 cells by the DEAE-dextran-chloroquine procedure (14). About 0.5×10^6 cells were transfected with 0.5 µg of covalently closed circular Py DNA. The cells were harvested 38 to 40 h posttransfection,

and Hirt extracts (9) were electrophoresed in 0.8% (wt/vol) agarose gels with 0.5 µg of ethidium bromide per ml. The results show a drastic decrease in DNA synthesis for the Py-*d*/B3 mutant compared with the wt (Fig. 2A). The quantitation of the relative DNA yields obtained by densitometric scanning is shown in Fig. 2B. A severalfold increase of the DNA input in these transfection experiments did not result in a significant change of the synthesis of wt or mutant DNA (data not shown). Therefore, artifactual results due to genome dosage can be ruled out. To rule out that the mutant DNA replication defect was due to modifications of the regulatory region accidentally introduced during the mutagenesis procedure, we replaced the whole region in the mutant with that of the wt by exchanging the *Bam*HI-*Bgl*II DNA fragment. Identical results to those reported above were obtained with this reconstructed genome. These data clearly demonstrate that the defect in mutant DNA replication is the direct consequence of the deletion of 1,166 base pairs in the late coding region.

To assess the *cis*-dominant character of this mutation, we cotransfected mutant and wt DNA into 3T6 cells. The results (Fig. 2C) show that the wt genome was unable to restore the replication competence of the cotransfected viral mutant. Furthermore, this lack of complementation between the mutant and the wt was not dependent on the relative ratio of the two DNA species.

The mutant phenotype is the direct consequence of the deletion of specific sequences. Two different explanations can be proposed for the drastically lower level of DNA synthesis observed in Py-*d*/B3. (i) The extensive deletion in the genome may produce some kind of steric constraint that prevents formation of a proper replicative genomic structure (3, 5, 18, 20). Therefore, the mutant genome would not constitute a suitable substrate for the replication machinery. (ii) The deletion may encompass specific sequences directly involved in the control of the viral replication process (12).

To distinguish between these hypotheses, we reconstructed the original genome size by inserting at the deletion site (i) an unrelated DNA fragment of 1,374 base pairs, obtained by digestion of pBR322 with *Sau*3A1 (Ins21); (ii) a fragment of the Py genome (nucleotides 5022 to 1192) containing an additional viral origin of replication, which was obtained by digestion of wt viral DNA with *Sau*3A1 and inserted in the same (Dori1) or the opposite (Dori2) orientation with respect to the resident origin of replication; (iii) the authentic viral deleted sequences either in the same (SPR1) or in the inverse (SPR2) polarity.

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FIG. 1. *Bal* 31 deletion in the Py late region. Some restriction sites are shown in order to locate more clearly the termini of the deletion. The nucleotide sequence on both sides of the *Bgl* linker is shown at the bottom. The Py physical map is redrawn from Griffin et al. (7).

The replication abilities of these constructs were assayed by transfection of 3T6 cells as previously reported (14). The results clearly demonstrate that a very good recovery of viral replication activity was attained only after insertion of the authentic Py deleted sequences (Fig. 3). Moreover, this effect was orientation independent. Therefore, these data indicate that the Py late sequences are involved directly in the positive control of viral DNA synthesis.

FIG. 2. Decrease in synthesis of Py DNA for viral mutant Py-dIB3 and assessment of the *cis*-acting character of the mutation. (A) Fluorograms of 3T6-transfected cells. Hirt extracts (9) were run on 0.8% agarose gels in the presence of 0.5 μ g of ethidium bromide per ml. Lanes: 1, mock transfection; 2, wt; 3, Py-dIB3. (B) Densitometric scanning of the gel shown in panel A. Scannings of all samples (in triplicate) were performed on an LKB Ultrascan XL; the standard error was $\pm 5\%$. Only the areas of the gels containing Py form I DNA were scanned. (C) Cotransfection of 3T6 cells with Py wt and Py-dIB3 mutant DNA. Lanes: 1, wt; 2, Py-dIB3; 3, wt plus Py-dIB3. The ratio between the two different DNA species was 0.1 (wt/mutant). Arrowheads indicate Py and deletion mutant form I DNA.

FIG. 3. Restoration of Py replication competence after reconstruction of the viral genome with different sequences. (A) Fluorograms obtained as for Fig. 2. (B) Densitometric scanning of the samples shown in panel A. The standard error was $\pm 5\%$. Lanes: 1, wt; 2, Py-dIB3; 3, Ins21; 4, Dor1; 5, Dor2; 6, SPR1; 7, SPR2; 8, mock transfection. See the text for a more detailed description of the viral constructs.

One of the mechanisms whereby this control could be exerted is via binding of a cellular regulatory factor(s) to these sequences. In order to investigate the existence of such a factor(s), we cotransfected wt Py DNA into 3T6 cells in the presence of increasing amounts of the viral genome fragment spanning nucleotides 4632 to 3466 (*Bam*HI site to *Hind*II site; Fig. 1) that was cloned in plasmid pBR322. The results of these experiments are shown in Fig. 4. The data demonstrate that the level of Py replication was severely inhibited by the presence of the cloned fragment, already at a molar ratio of wt DNA to cloned fragment equal to 1. Moreover, the reduction of Py DNA synthesis was not caused by the cotransfected pBR322 sequences, since the plasmid per se had no effect on the viral replication level (Fig. 4C). These results are consistent with the presence of a cellular regulatory factor(s) that binds to the Py late region sequences and is essential for the replication of the viral genome. The titration of this putative replication factor by the cotransfected viral late region fragment leads to the complete abolition of wt DNA replication. Preliminary evi-

FIG. 4. Abolition of Py replication after cotransfection with the *Bam*-*Bgl* DNA fragment. (A) Fluorograms obtained as for Fig. 2 after cotransfection of 3T6 cells at the indicated molar ratios of the *Bam*-*Bgl* fragment to wt DNA. (B) Densitometric scanning of the samples shown in panel A. The standard error was $\pm 5\%$. Lanes: 1, wt; 2, 3, and 4, Py DNA plus viral fragment at the indicated molar ratios; 5, Py-dIB3; 6, mock transfection. (C) Transfection by Py DNA in the presence of plasmid pBR322. Lanes: 1, Py DNA; 2, Py and pBR322 DNA; 3, pBR322 DNA. The ratio between the two DNA species was 0.1 (Py/pBR322).

dence, obtained in our laboratory by gel retardation and DNA-/protein-binding assays, suggests that nuclear extracts of uninfected 3T6 cells contain proteins that bind to these sequences of the Py late region. Experiments are in progress to characterize this protein factor controlling viral DNA synthesis and to map its specific binding site.

The chromatin and genetic organization of Py and of the closely related monkey virus simian virus 40 have been thoroughly studied in an effort to clarify the structure-function relationships of these viral genomes (2, 8, 11, 19). A recent report (4) describes a Py mutation mapping in the late region that confers a growth advantage to the mutant over the wt. Furthermore, we have preliminary results showing that one or more DNase I-sensitive sites are present in this area of the Py late region. The importance of such sites in gene expression and function is generally recognized (6). There is also evidence that these late sequences play a role in establishing Py replication competence in different host cells (L. Ricci et al., manuscript in preparation). Finally, the simian virus 40 capsid protein VP1 was recently shown to be involved in the spacing of nucleosomes in viral minichromosomes (1). This finding suggests a role in chromatin assembly for a late region gene product. However, to our knowledge, this is the first report demonstrating the direct involvement of the Py late region in the functional control of viral genome replication.

In conclusion, the data presented here show that Py late coding sequences do not simply specify the information for capsid proteins but must contain elements of regulatory and functional significance.

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LITERATURE CITED

- Blasquez, V., A. Stein, C. Ambrose, and M. Bina. 1986. Simian virus 40 protein VP1 is involved in spacing nucleosomes in minichromosomes. *J. Mol. Biol.* **191**:97-102.
- Caruso, M., A. Felsani, and P. Amati. 1986. Sites hypersensitive to, and protected from, nuclease digestion in the regulatory region of wild-type and mutant polyomavirus chromatin. *EMBO J.* **5**:3539-3546.
- De Pamphilis, M. L. 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. *Cell* **52**:635-638.
- De Simone, V., G. La Mantia, L. Lania, and P. Amati. 1985. Polyomavirus mutation that confers a cell-specific *cis* advantage for viral DNA replication. *Mol. Cell. Biol.* **5**:2142-2146.
- Drew, H. R., and A. A. Travers. 1985. DNA bending and its relation to nucleosome positioning. *J. Mol. Biol.* **186**:773-790.
- Eissenberg, J. C., I. L. Cartwright, G. H. Thomas, and S. C. R. Elgin. 1985. Selected topics in chromatin structure. *Annu. Rev. Genet.* **19**:485-536.
- Griffin, B. E., E. Soeda, B. G. Barrell, and R. Staden. 1980. Sequence and analysis of polyoma virus DNA, p. 843-910. *In* J. Tooze (ed.), *Molecular biology of tumor viruses: DNA tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Herbomel, P., S. Saragosti, D. Blangy, and M. Yaniv. 1981. Fine structure of the origin-proximal DNase I-hypersensitive region in wild-type and EC mutant polyoma. *Cell* **25**:651-658.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
- Jones, N. C., W. J. Rigby, and E. B. Ziff. 1988. *trans*-Acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. *Genes Dev.* **2**:267-281.
- Jongstra, J., T. L. Reudelhuber, P. Oudet, C. Benoist, C. B. Chae, J.-M. Jeltsch, B. Mathis, and P. Chambon. 1984. Induction of altered chromatin structures by simian virus 40 enhancer and promoter elements. *Nature (London)* **307**:708-714.
- Kern, F. G., S. Pellegrini, and C. Basilico. 1986. *cis*- and *trans*-acting factors regulating gene expression from the polyomavirus late promoter, p. 115-124. *In* M. Botchan et al. (ed.), *Cancer cells*, vol. 4. DNA tumor viruses: control of gene expression and replication. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Luthman, H., M.-G. Nilsson, and G. Magnusson. 1982. Non-contiguous segments of the polyoma genome required in *cis* for DNA replication. *J. Mol. Biol.* **161**:533-550.
- Luthman, H., and G. Magnusson. 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11**:1295-1308.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maxam, A. M., and W. Gilbert. 1974. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* **74**:560-564.
- Tooze, J. (ed.). 1980. *Molecular biology of tumor viruses: DNA tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Trifonov, E. N. 1985. Curved DNA. *Crit. Rev. Biochem.* **19**:89-106.
- Watson, J. B., and J. D. Gralla. 1987. Simian virus 40 associates with nuclear superstructures at early times of infection. *J. Virol.* **61**:748-754.
- Weintraub, H. 1985. Assembly and propagation of repressed and derepressed chromosomal states. *Cell* **42**:705-711.