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trans-Activation of Parvovirus P_{38} Promoter by the 76K Noncapsid Protein

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The autonomously replicating parvoviruses contain a 5-kilobase linear single-stranded DNA genome that produces two noncapsid proteins, N1 and N2, and two overlapping capsid proteins, VP1 and VP2. To characterize the regulation of viral transcription, we began with a study of the promoter for the coat proteins (P_{38}) at map unit 38. Various constructions containing the P_{38} promoter were fused to the bacterial gene for chloramphenicol acetyltransferase (*cat*), and the relative efficiency of expression was determined in the presence and absence of parvovirus gene products. Our results show that the P_{38} promoter is a weak promoter without a *trans*-activation mediated by the 76,000-molecular-weight (76K) N1 protein. The N1 protein, supplied either by superinfection with virus or cotransfection with the cloned N1 gene, increased greatly the expression of the P_{38} promoter. In addition, sequences 3′ to the promoter, within the region +127 to +648 (assuming an mRNA start site at 2008), were required for optimal expression but not for *trans*-activation. These results suggest that the production of parvovirus capsid proteins is under the indirect control of the P_4 promoter and one of its gene products.

The rodent parvoviruses, which include the minute virus of mice, H-1, H-3, and Kilham rat virus, have been studied in the most detail. Two of these, minute virus of mice and H1, have been completely sequenced (1, 24). The analyses of the viral DNA sequences, hybrid-arrested translation (24), and hybrid-selected translation (4) experiments as well as direct analysis of the proteins (17, 29) provide a genetic map that is summarized in Fig. 1.

Mapping of viral RNA has placed the locations of promoters at map positions 4 and 38 (6, 18, 28). These sites contain the canonical TATA boxes, and at 38 there is also the sequence CCAAT, which is commonly found at eucaryotic promoters (2). In most studies, the appearances of the noncapsid protein N1 (also called NCVP1 or NS1) and the coat proteins are nearly simultaneous (4, 15). The plasmids shown were constructed by standard methods (12) with enzymes obtained from commercial sources. The plasmids pSV2cat and pA_{10}cat were obtained from L. Laimins (National Institutes of Health). Replicative form (RF) DNA of H-1 was isolated as previously described (20). The H-1 fragments *Bcl*I-*Pst*I (nucleotides 1661 to 2132) and *Eco*RI-*Pst*I (nucleotides 1088 to 2132) were cloned in the vector pUC8. They were then recovered as an *Eco*RI-*Hin*dIII fragment, with the *Hin*dIII site coming from the polylinker of the vector, and recloned into pBR327 to give pH1 and pH2, respectively. The *cat* gene, the simian virus 40 (SV40) small-T splice region, and the poly(A) signal were obtained from pA_{10}cat as a *Hin*dIII-*Bam*HI fragment and cloned into those sites of pH1 and pH2, giving pH1cat and pH2cat. The pH3cat plasmid was constructed by inserting the *Pst*I-*Hin*dIII fragment (nucleotides 2132 to 2651) obtained from H-1 RF DNA into pH1cat that had been restricted with *Hin*dIII and partially restricted with *Pst*I. pH5cat was constructed by inserting a *Hin*dIII fragment containing the H-1 sequences from 0+ to 2651 into the *Hin*dIII site of pBR327cat. pBR327cat was made by cloning the cat *Hin*dIII-*Bam*HI fragment into those sites in pBR327. The H-1 fragment containing the entire left end of H-1 was obtained from pSR1, a genomic clone of *ts*6 H-1 in the vector pUC9. pSR1 was constructed by treating about 4 μg of *ts*6 RF DNA with 2 U of *Bal*31 for 2 min at 37°C, blunting the ends with T4 DNA polymerase, and ligating *Bam*HI linkers to the ends. After restriction with *Bam*HI, the DNA was purified further by gel electrophoresis and ligated to *Bam*HI-cleaved pUC9. Two of the clones isolated, pSR1 and pSR2, have genomic length inserts of H-1 DNA; one is inverted with respect to the other. The *Hin*dIII site in pSR1 flanking the zero position of H-1 is from the polylinker of the vector. pH6cat was constructed from pH5cat by restricting it with *Bgl*II, which cleaves H-1 at nucleotide 1246, filling in the restriction site with the Klenow fragment of pol 1, and religating the plasmid. This will produce a 4-base insertion in the N1 gene (but not N2), and the resulting frameshift will cause translation of N1 to terminate at nucleotide 1286, giving a truncated N1 of about 38K. Plasmids were prepared after chloramphenicol amplification in *Escherichia coli* HB101 or TB1 (pUC8 or pUC9 vectors) as previously described (23). The cells hosting the plasmids pH2cat, pH5cat, and pH6cat are resistant to chloramphenicol at 170 μg/ml due to procaryotic promoter activity between H-1 nucleotides 1088 and 1661, so they were amplified with spectinomycin at 300 μg/ml.

In this study, various H-1 RF DNA fragments containing the P_{38} promoter were fused to the chloramphenicol acetyltransferase (*cat*) gene, and their activity was assessed in transient expression assays (5, 11, 25). The constructions include the TATA box of the P_{38} promoter and extend in both the 5′ and 3′ directions (Fig. 1). The breakpoint between the H-1 sequences on the 3′ side of the promoter were at the *Pst*I site at map position 41 or the *Hin*dIII site at map unit 51. These plasmids were assayed for expression of chloramphenicol acetyltransferase (CAT) activity 44 h after transfection by the calcium phosphate precipitation method (32) (Table 1). Transfections were in replicate cultures of NB cells made by seeding 100-mm petri dishes with 5×10^5 cells per dish. The following day the medium was changed 2 to 4 h before transfection. The cells were transfected with 5 μg of plasmid DNA per dish in the presence of carrier salmon sperm DNA at 20 μg/ml by the calcium phosphate precipi-

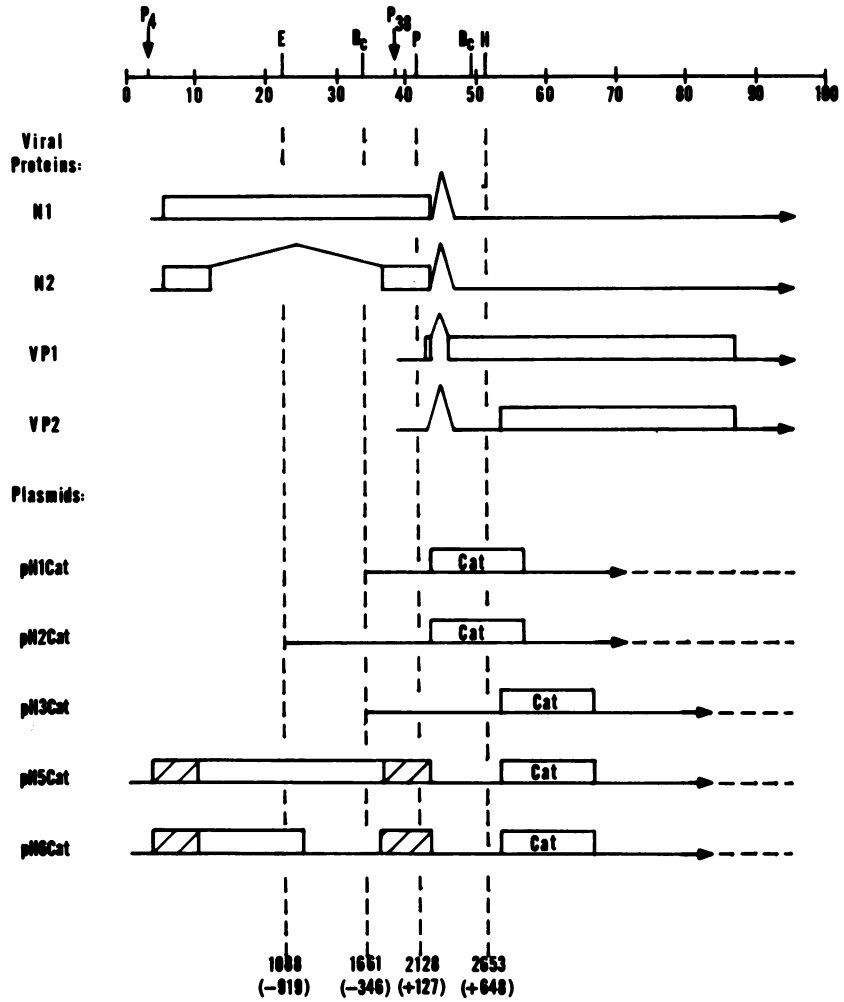

FIG. 1. Genetic map of autonomously replicating parvoviruses and plasmids containing the late promoter. The promoters at map units 4 and 38 are indicated by P_4 and P_{38}, respectively. The restriction enzyme sites are: E, *Eco*RI; Bc, *Bcl*I; P, *Pst*I; and H, *Hin*dIII. The numbers below the line are map units, with 0 at the 3′ end of viral plus strand DNA. The exons are indicated as the open blocks. The reading frames for N1 and N2 are the same. The reading frames for the VP1 exon 2 and the VP2 exon are also the same. The numbers refer to the nucleotide number in the H-1 genome (24), and the numbers in parentheses refer to the nucleotide position with respect to the late message initiation site assumed to be at nucleotide 2008 (1).

tation method as described by Wigler et al. (32). The cultures were given a glycerol shock with 15% glycerol for 4 min at room temperature at 4 h after transfection. The cultures superinfected with H-1 virus were infected at a multiplicity of infection of 5 to 10 PFU per cell 24 h posttransfection. For the CAT assay, the cultures were washed once with Hanks balanced salts and collected with the aid of a rubber policeman in 1 ml of Hanks balanced salts. The cells were pelleted in a microfuge for 2 min at room temperature and suspended in 100 μl of 0.25 M Tris (pH 7.8) and enzyme extracted by three cycles of freeze-thawing as described previously (11). The extracts were clarified by 15 min of centrifugation in a microfuge at 4°C, and 60 μl was used to assay for CAT as described previously (5). The acetylated forms of [^{14}C]chloramphenicol were separated from the nonacetylated form by chromatography on silica gel plates with 95% chloroform and 5% methanol. After autoradiography, the thin-layer chromatogram was quantitated by densitometry of the autoradiograph with an LKB scanning densitometer. The plasmids pH1cat and pH2cat (Fig. 1), which have +127 as the 3′ boundary of their H-1 sequences, had little constitutive activity, but pH3cat (which extends to +648) showed a very modest improvement. Superinfection with H-1 virus of cells transfected with these plasmids or cotransfection with

TABLE 1. Expression of CAT by various plasmids in a transient assay in NB cells[a]

Plasmid	% Converted	Normalized to pH1cat
pH1cat	1.1	1
pH1cat + H-1	24.8	23
pH2cat	0.6	0.6
pH2cat + H-1	20.4	19
pH3cat	3.8	3
pH3cat + H-1	39.6	36
pSV2cat	49.3	45
pSV2cat + H-1	35.9	33

[a] Reference 26.

genomic clones of H-1 (Table 2) caused a significant increase in expression of CAT. The highest level of expression was achieved by pH3cat. When the superinfection was carried out with the temperature-sensitive mutants of H-1, *ts*2, and *ts*6, the *trans*-activation occurred at the restrictive temperature of 39.5°C (*ts*2 data not shown) (Table 2). The mutant *ts*6 is indistinguishable from *ts*1, and both *ts*2 and *ts*6 are capsid protein mutants (21). This provided presumptive evidence that the function required in *trans* may be supplied by a noncapsid protein. Similarly, the related viruses Lu111 and canine parvovirus (CPV2) were also positive for the *trans*-activation of the pH3cat plasmid (data not shown). This is of particular interest, because Lu111 did not complement an H-1 deletion mutant defective for RF DNA replication (22). CPV2 shows a high degree of homology to H-1 in the structural gene sequences (both the carboxy-terminal part of N1 and the amino-terminal part of VP1) in the region containing the late promoter, but it does diverge for sequences within the intron region (22a). CPV2 was tested for complementation for *trans*-activation in DK cells (16) (SV40-transformed dog kidney) which are permissive for both CPV and H-1 lytic infections.

The expression of CAT by the plasmids pH1cat or pH2cat was enhanced by superinfection with H-1, but to a smaller absolute level than by pH3cat. The extension of the promoter containing sequences in the 5′ direction from the *Bcl*I site in pH1cat to the *Eco*RI site in pH2cat did not improve the expression. The plasmid pSV2cat, which is similar to the parvovirus constructions, except that it directs CAT synthesis with the unrelated SV40 early promoter, showed no enhancement of expression after superinfection with H-1; rather, it declined. These results argue that the enhancement has specificity and that sequences between −350 and +127 are required for the effect.

We have sought sequences in the H-1 genome that might have enhancer activity and have cloned a number of regions into the *Bgl*II site of pA_{10}cat (5, 25). pA_{10}cat has the cat gene fused to the SV40 early promoter from which the enhancer sequences have been deleted. The addition of enhancer sequences to pA_{10}cat may increase the expression of cat so that this plasmid can be used to detect enhancer elements (25). We found no enhancement with fragments containing noncoding regions from map units 79 to 98 and 0.9 to 9 (data not shown).

To test the noncapsid proteins coded by the left half of the parvovirus genome for the *trans*-activation of the late promoter, we constructed a plasmid containing only the noncapsid genes with the complete left half of the virus from the genomic clone pSR1, which was active for *trans*-activation by cotransfection. The region for the capsid proteins from map unit 51 to their end is deleted in these constructions. We also introduced a frameshift mutation in the N1 gene by filling in the *Bgl*II site at map position 23 in pH5cat, making the plasmid pH6cat (Fig. 1). This insertion should produce a truncated N1 protein of about 38K and not affect the N2 protein. The expression of CAT by these plasmids is shown in Table 3. pH5cat expressed CAT at a level approximately 100 times that of pH6cat. pH6cat was reduced to a level equivalent to the constitutive level of pH3cat in the absence of *trans*-activation. Both pH3cat and pH6cat responded to *trans*-activation by contransfection with the genomic clone pSR2, therefore the mutation in pH6cat abolished the capacity of pH6cat to *trans*-activate itself but not its ability to respond to *trans*-activation. This identifies the N1 gene as responsible for the *trans*-activation of the late promoter.

The mechanism of action of the N1-mediated enhancement of expression is unknown. Since the parvoviruses Lu111 and CPV2 complement for this function, the N1 protein and its target sequences appear to be highly conserved in the autonomously replicating parvoviruses. We have not determined whether the enhanced CAT expression is due to increased transcription or to a later event, such as altered mRNA processing. Experiments are in progress to distinguish between these possibilities.

The N1 protein is a phosphoprotein that is detectable by immunoprecipitation at about the same time as capsid protein synthesis (4, 15). The results described here suggest that N1 plays a role in maximizing expression of the P_{38} promoter, which directs the capsid protein transcripts. Therefore, it is possible that the P_4 promoter is an early promoter and the P_{38} promoter is a late promoter, in analogy to papovaviruses and adenoviruses (8, 10, 14). We are conducting further studies to determine whether the N1 protein *trans*-activates its own early promoter and are mappng the precise sequences required for the *trans*-activation of the late promoter. Parvoviruses are organized into a tandemly arranged transcriptional complex with a noncapsid gene that is 5′ to a capsid gene that has its promoter embedded within the early gene. This represents a remarkable conservation of space, and the N1 gene-late promoter may make a useful eucaryotic expression system. The autonomously replicating parvoviruses require a *trans*-acting function for their RF DNA replication (13, 22). A mutant minute virus of mice

TABLE 2. Densitometry of CAT expression by pH3cat-transfected NB cultures[a]

No.	Culture	% Converted	Normalized to pH3cat
1	pH3cat	<0.3	1
2	pH3cat + wild-type H-1	4.9	>14
3	pH3cat + *ts*6 (39.5°C)	4.1	>12
4	pH3cat + *ts*6 (32°C)	2.1	>6
5	pH3cat + pSR1	15.6	>45
6	pH3cat + pSR2	16.2	>47

[a] Cultures were superinfected with wild-type H-1 (culture 2) or *ts*6 at either 39.5°C 32 h posttransfection (culture 3) or 32°C 30 postransfection (culture 4) at a multiplicity of infection 3 to 5 PFU per cell. Cultures 5 and 6 were cotransfected with 5 μg of the genomic clones of *ts*6 H-1, pSR1, and pSR2 at 37°C. The cultures were harvested at 48 h postransfection for assay of CAT. Acetylated chloramphenicol was below detection for pH3cat at the exposure used for this assay; based on other samples on this plate it could be estimated that the percentage converted was less than 0.3.

TABLE 3. Densitometry of CAT expression by various plasmids[a]

No.	Culture	% Converted	Normalized to pH3cat
1	pH1cat	<0.5	1
2	pH3cat	<0.5	1
3	pH3cat + pSR2	14	>28
4	pH5cat	48	>96
5	pH5cat + pSR2	36	>72
6	pH6cat	<0.5	1
7	pH6cat + pSR2	16	>32

[a] Densitometry was as described for Table 2. The acetylated CAT produced by pH1cat, pH3cat, and pH6cat (cultures 1, 2, and 6) was below the level detectable with this exposure. Similar results were obtained in another experiment. In both cases, coinfection of pH5cat with pSR2 showed a moderate reduction in the CAT expressed. This may show competition for a rate-limiting factor.

deleted in the capsid gene is not defective in RF DNA replication, suggesting that either N1, N2, or both are required for DNA replication (13). Similar results have been obtained for adeno-associated viruses (7, 30). During the course of this work, it was reported that deletion mutants of adeno-associated viruses demonstrated a similar dependence on a noncapsid protein for late-promoter transcription (M. Labow, P. Hermonat, N. Muzyczka, and K. I. Berns. Annu. Meet. Am. Soc. Virol., August 1984).

Thus, parvoviruses join an expanding group of animal viruses that regulate the transcription of their late genes with a protein product of a gene expressed early in infection. This list includes papovaviruses (3, 9), adenoviruses (14), herpesviruses (19), and certain retroviruses (27). This strategy is one that has been well studied in various bacteriophages (31). In this light, DNA animal viruses may be obliged to secure expression of only one early gene with the host cell transcription machinery before they can assume mastery over the expression of the rest of their genetic program.

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LITERATURE CITED

1. **Astell, C. R., M. Thomson, M. Merchlinsky, and D. C. Ward.** 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. Nucleic Acids Res. **11**:999–1018.
2. **Bensimhon, M., J. Gabarro-Arpa, R. Ehrlich, and C. Reiss.** 1983. Physical characteristics in eucaryotic promoters. Nucleic Acids Res. **11**:4521–4540.
3. **Brady, J., J. B. Bolen, M. Radonovich, N. Salzman, and G. Khoury.** 1984. Stimulation of simian virus 40 late gene expression by simian virus 40 tumor antigen. Proc. Natl. Acad. Sci. U.S.A. **81**:2040–2044.
4. **Cotsmore, S. F., L. J. Sturzenbecker, and P. Tattersall.** 1983. The autonomous parvovirus MVM encodes two nonstructural proteins in addition to its capsid polypeptides. Virology **129**:333–343.
5. **Gorman, C. M., L. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
6. **Green, M. R., R. M. Lebovitz, and R. G. Roeder.** 1979. Expression of the autonomous parvovirus H1 genome: evidence for a single transcriptional unit and multiple spliced polyadenylated transcripts. Cell **17**:967–977.
7. **Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka.** 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol. **51**:329–339.
8. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. U.S.A. **76**:3665–3669.
9. **Keller, J. M., and J. C. Alwine.** 1984. Activation of the SV_{40} late promoter: direct effects of T antigen in the absence of viral DNA replication. Cell **36**:381–389.
10. **Khoury, G., and E. May.** 1977. Regulation of early and late simian virus 40 transcription: overproduction of early viral RNA in the absence of a functional T-antigen. J. Virol. **23**:167–176.
11. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Merchlinsky, M. J., P. J. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. **47**:227–232.
14. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**:213–220.
15. **Paradiso, P. R.** 1984. Identification of multiple forms of the noncapsid parvovirus protein NCVP1 in H-1 parvovirus-infected cells. J. Virol. **52**:82–87.
16. **Paradiso, P. R., S. L. Rhode III, and I. I. Singer.** 1982. Canine parvovirus: a biochemical and ultrastructural characterization. J. Gen. Virol. **62**:113–125.
17. **Paradiso, P. R., K. R. Williams, and R. L. Costantino.** 1984. Mapping of the amino terminus of the H-1 parvovirus major capsid protein. J. Virol. **52**:77–81.
18. **Pintel, D., D. Dadachanji, C. R. Astell, and D. C. Ward.** 1983. The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. Nucleic Acids Res. **11**:1019–1038.
19. **Post, L., S. Mackem, and B. Roizman.** 1981. Regulation of alpha genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. Cell **24**:555–566.
20. **Rhode, S. L., III.** 1974. Replication process of the parvovirus H-1. II. Isolation and characterization of H-1 replicative form DNA. J. Virol. **13**:400–410.
21. **Rhode, S. L., III.** 1976. Replication process of the parvovirus H-1. V. Isolation and characterization of temperature-sensitive H-1 mutants defective in progeny DNA synthesis. J. Virol. **17**:659–667.
22. **Rhode, S. L., III.** 1982. Complementation for replicative form DNA replication of a deletion mutant of H-1 by various parvoviruses. J. Virol. **42**:1118–1122.

22a. **Rhode, S. L., III.** 1985. Nucleotide sequence of the coat protein gene of canine parvovirus. J. Virol. **54**:630–633.

23. **Rhode, S. L., III, and B. Klaassen.** 1982. DNA sequence of the 5′ terminus containing the replication origin of parvovirus replicative form DNA. J. Virol. **41**:990–999.
24. **Rhode, S. L., III, and P. R. Paradiso.** 1983. Parvovirus genome: nucleotide sequence of H-1 and mapping of its genes by hybrid-arrested translation. J. Virol. **45**:173–184.
25. **Rosenthal, N., M. Kress, P. Gruss, and G. Khoury.** 1983. BK viral enhancer element and a human cellular homolog. Science **222**:749–755.
26. **Shein, J. M., and J. F. Enders.** 1962. Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. Proc. Soc. Exp. Biol. Med. **109**:495–500.
27. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. Transacting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **225**:381–385.
28. **Tal, J., D. Ron, P. Tattersall, S. Bratosin, and Y. Aloni.** 1979. About 30% of minute virus of mice RNA is spliced out following polyadenylation. Nature (London) **279**:649–651.
29. **Tattersall, P. A., A. J. Shatkin, and D. C. Ward.** 1977. Sequence homology between the structural polypeptides coded for by minute virus of mice. J. Mol. Biol. **111**:375–394.
30. **Tratschin, J.-D., I. L. Miller, and B. J. Carter.** 1984. Genetic analysis of adeno-associated virus: properties of deletion mutants constructed in vitro and evidence for an adeno-associated virus replication function. J. Virol. **51**:611–619.
31. **von Hippel, P. H., D. G. Bear, W. D. Morgan, and J. A. McSwiggen.** 1984. Protein-nucleic acid interactions in transcription: a molecular analysis. Annu. Rev. Biochem. **53**:389–446.
32. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaut, and L. Chasin.** 1979. Biochemical transfer of a single copy eukaryotic genes using total cellular DNA as donor. Proc. Natl. Acad. Sci. U.S.A. **76**:1373–1376.