10. W. Trager *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6527 (1981).
11. Isolate NF54 was derived from a patient living near Schipol Airport, Amsterdam, who had never left the Netherlands [see T. Ponnudurai, A. D. E. M. Leeuwenberg, J. H. E. Th. Meuwissen, *Trop. Geogr. Med.* **33**, 50 (1981)]. Clone 3D7 was derived from NF54 by limiting dilution (*9*). NF54 was cultured in a flask (*20*) to a parasitemia of approximately 0.5%. The culture was then shaken gently for 48 hours at 37°C so that most parasitized erythrocytes (about 90%) contained only single parasites. Dilutions were made until there was an average of 0.5 parasite per 0.1 ml of culture. Portions (0.1 ml) were placed in individual wells of 96-well microtiter plates and maintained in an incubator (5% CO_2, 5% O_2, 90% N_2). Medium was changed every 48 hours, and new erythrocytes, in a volume equal to that in each well, were added every 6 days. Eight out of 20 wells showed parasites after 14 days. The contents of one well, denoted 3D7, were grown in a flask and recloned by the same procedure. Two out of 20 wells contained parasites after 11 days. The contents of one well were taken for use as the 3D7 clone.
12. Clone HB3 was derived from isolate H1 from Honduras by W. Trager by microscopic selection.
13. Pyrimethamine response was determined by the method of S. Thaithong and G. H. Beale [*Trans. R. Soc. Trop. Med. Hyg.* **75**, 271 (1981)]. Parasites were cultured in microtiter plates and exposed to pyrimethamine at doses of $10^{-9}M$ to $10^{-5}M$ for 72 hours with daily changes of medium with drug. Giemsa-stained blood smears were examined to determine the minimum inhibiting concentration (MIC) of drug that killed all, or nearly all, the parasites. The MIC for 3D7 was $10^{-7}M$; for the purpose of this work, parasites with this MIC are classified as pyrimethamine-sensitive. For HB3, the MIC was $10^{-5}M$, and these parasites are classified as resistant.
14. Parasites were examined for electrophoretic forms of ADA by the method of A. Sanderson, D. Walliker, and J.-F. Molez [*Trans. R. Soc. Trop. Med. Hyg.* **75**, 263 (1981)], with the use of agarose gel electrophoresis (Corning) instead of starch gel electrophoresis; the banding patterns of ADA variants are similar in the two systems. Parasites were freed from host erythrocytes by lysis in saponin, concentrated by centrifugation, and disrupted by freezing and thawing prior to electrophoresis (for 25 minutes). The electrophoresis buffer was 0.05*M* sodium barbital, *p*H 8.6.
15. R. Hall *et al.*, *Mol. Biochem. Parasitol.* **7**, 247 (1983); J. S. McBride, C. I. Newbold, R. Anand, *J. Exp. Med.* **161**, 160 (1985); J. S. McBride, P. D. Welsby, D. Walliker, *Trans. R. Soc. Trop. Med. Hyg.* **78**, 32 (1984); J. S. McBride and C. Wilson, personal communication. The 195-kD and 40-kD antigens are associated with late-stage trophozoites and schizonts. Variant forms of these antigens were identified by indirect IFA, as described by J. S. McBride, D. Walliker, and G. Morgan [*Science* **217**, 254 (1982)]. Cultures containing schizonts were washed three times in RPMI 1640 medium without serum, and pipetted onto wells of multispot microscope slides so that each well contained approximately 10^4 schizonts. The preparations were dried, fixed in acetone, and stained with Mabs 7.3, 9.2, or 12.3, and then incubated with fluorescein isothiocyanate–conjugated antiserum to mouse immunoglobulin G (Sigma). Preparations were examined by ultraviolet microscopy. Wells contained either brightly fluorescing organisms that were scored +, or no fluorescing organisms that were scored −.
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21. Cultures of *P. falciparum* were maintained in flasks by using modifications of the methods of Trager and Jensen and of Haynes *et al.* [see (*6*)]. For routine maintenance, cultures were kept in fresh human erythrocytes, type O, at 5% hematocrit in RPMI 1640 medium containing 10% human serum, with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Medium was changed daily, and fresh erythrocytes were added twice weekly. For gametocytes, cultures were established at a parasitemia of approximately 0.3% at 6% hematocrit. When the parasitemia had attained 5%, about 4 days later, the hematocrit was lowered to 3%. Cultures were then maintained without addition of further erythrocytes, but with daily changes of medium until day 15, when mature gametocytes were present.
22. L. S. Ozaki, R. W. Gwadz, G. N. Godson, *J. Parasitol.* **70**, 831 (1984).
23. PFG gel electrophoresis was carried out according to the system of G. F. Carle and M. V. Olson [*Nucleic Acids Res.* **12**, 5647 (1984)]. Parasites were prepared in agarose blocks that were incubated in a mixture of tris, EDTA, Sarkosyl, and Proteinase K for 48 hours as described by Kemp *et al.* (*19*) before insertion into the gel. The gel was 1.5% agarose (Seakem ME) in 0.5× tris-borate-EDTA (TBE) buffer. Electrophoresis was carried out at 300 V for 18 hours at 14° to 16°C with a pulse time of 75 seconds.
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27. Chromosomal DNA was extracted from PFG gel by a variation of the procedure of B. Vogelstein and D. Gillespie [*Proc. Natl. Acad. Sci. U.S.A.* **76**, 615 (1979)]. Agarose slices were dissolved in four volumes (by weight) of a mixture of 6*M* $NaClO_4$, 10 m*M* tris acetate, 1 m*M* EDTA, *p*H 8.0 (PTE), at 37°C. The DNA in solution was sheared twice by passing it through a 30-gauge needle. A 25% suspension (25 μl) of borosilicate glass powder was mixed with the solution for 2 hours on a rocking platform. The glass powder with bound DNA was recovered by centrifugation. After washes with PTE, isopropyl alcohol, and 100% ethanol, the powder was dried briefly in the open tube. DNA was eluted from the glass into two 50-μl washes of 10 m*M* tris acetate and 1 m*M* EDTA, *p*H 8.0, which were combined and passed through a Sephadex G-50 column.
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30. We thank F. E. Neva, L. H. Miller, and W. E. Collins for advice on the design of this project; T. Ponnudurai for providing isolate NF54; P. Nguyen-Dinh for making available clone HB3; J. S. McBride for monoclonal antibodies; N. Papadopoulos and R. Costello for assistance with enzyme electrophoresis; and J. Dvorak for photographic assistance. Supported by the Medical Research Council of Great Britain (D.W.), and the United Nations Development Programme/World Bank, WHO Special Programme for Research and Training in Tropical Diseases (I.A.Q.).

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A Promoter with an Internal Regulatory Domain Is Part of the Origin of Replication in BPV-1

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Extrachromosomal elements that are stably maintained at a constant copy number through cell doublings are a good model system for the study of the regulation of DNA replication in higher eukaryotes. Previous studies have defined both cis and trans functions required for the regulated plasmid replication of the bovine papilloma virus in stably transformed cells. Here, a sequence known to be a cis-dominant element of the replication origin of the plasmid is shown to contain a promoter for transcription. Both in vitro and in vivo assays have been used to define this promoter and show that a sequence located just 3′ to the transcriptional start site is required for activity. This DNA sequence element, which has been defined through deletions, coincides with a binding site for a cellular factor and is also required for a functional origin of replication. Possible models for how a transcription factor may play a role in the regulation of DNA replication are discussed.

THE MECHANISMS BY WHICH THE eukaryotic DNA replication process is regulated are unknown (*1*). In particular, the structural features of a functional origin of DNA replication in higher eukaryotes and the nature of the factors that govern its activity have not been explored. However, the process of DNA replication is probably regulated at several different levels since it is a central part of the events in the cell cycle that commit the cell to divide. At present, virtually everything that is known about the process and the requirements for initiation of replication stems from studies of viral systems (*2*). Chromosomes from latent viruses that replicate as nuclear plasmids provide a model system in that their DNA synthesis must be regulated in order to maintain a constant copy number per nucleus. We are studying the regulation of bovine papilloma virus (BPV) DNA replication since this replicon appears to be intricately regulated to ensure orderly replication (*3, 4*). The viral components required for replication of BPV have been identified genetically (*5*). Two viral genes encoded in part by a single open reading frame (ORF) are required in the replication events (*6*). The R gene, which is encoded from the 3′ part of the E1 ORF acts in a positive way on replication, while the M gene, which is encoded by the 5′ part of the E1 ORF, serves as a negative regulator of DNA replication (*4, 6, 7*). It has been suggested that the activity of the M gene is what ensures the regulated and stable copy number of the DNA in transformed cells (*4, 7*).

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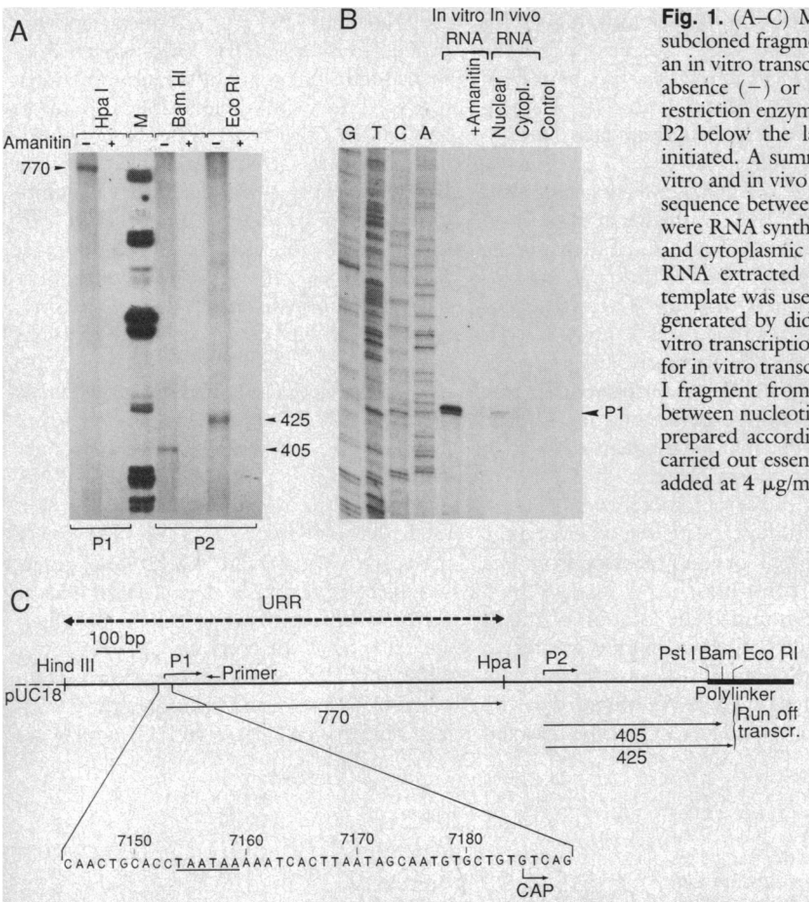

Fig. 1. (A–C) Mapping of the site of initiation for the P1 promoter. (**A**) The subcloned fragment of BPV shown in (C) was assayed for promoter activity with an in vitro transcription system. The gel shows runoff transcripts generated in the absence (−) or presence (+) of α-amanitin by digestion of the template with restriction enzymes Hpa I, Bam HI, and Eco RI. Lane M is a size marker. P1 and P2 below the lanes indicate at which promoter the runoff transcripts were initiated. A summary of the results is shown in (**C**). (**B**) Primer extension of in vitro and in vivo RNA. A synthetic oligonucleotide primer complementary to the sequence between 7284 and 7305 was used for primer extension. The templates were RNA synthesized in vitro in the absence or presence of α-amanitin. Nuclear and cytoplasmic RNA was isolated from ID13 cells. In the lane labeled control, RNA extracted from an in vitro transcription reaction without added DNA template was used for extension. The sequence ladder that serves as a marker was generated by dideoxy sequencing (*31*) of the plasmid used as a template for in vitro transcription, with the same primer used above. The template plasmid used for in vitro transcription assays was constructed by inserting a 1.5-kb Hind III–Pst I fragment from BPV into the polylinker of pUC18. The fragment is located between nucleotides 6958 and 471 in the BPV genome. Whole-cell extracts were prepared according to Manley *et al.* (*32*), and runoff transcription assays were carried out essentially as described (*33*) except that α-amanitin, where used, was added at 4 μg/ml. Primer extension analysis was carried out as described (*34*).

The BPV genome contains two cis-acting sequence elements, PMS1 and PMS2, which can establish marker DNA's as stable plasmids (*8*). Electron microscopic analysis indicates that the region of the viral genome containing PMS1 is a site for initiation of DNA replication (*9*). The cis-acting region defining PMS1 is itself genetically complex, and mutational analysis of the PMS1 region has revealed at least two essential sequence domains (*10*). Domain 2 is defined as the core element for DNA replication, while the upstream domain, domain 1, has been defined as a "replication enhancer" in that it functions in a position- and orientation-independent manner and can be replaced by other well-characterized viral enhancers (*10*).

We now describe a promoter that is embedded in the core origin domain of PMS1. Small deletions, which define the core origin of replication, also define regulatory elements for the promoter. It seems possible that a subset of factors required for transcription also have a function for initiation of replication. Furthermore, the promoter has an absolute requirement for sequences downstream of the cap site for function both in vivo and in vitro.

In transformed cells harboring BPV plasmids, a complex set of transcripts all derive from one strand that also encodes the eight early open reading frames (*11, 12*). A major previously identified promoter, designated P2, lies just upstream of these open reading frames. It defines the 3′ border of an approximately 1-kb-long region called the upstream regulatory region, URR, that contains regulatory elements shown to be important both for replication and transcription (*10, 13*). (See diagrams in Figs. 1 and 4.)

The existence of occasional truncated complementary DNA's (cDNA's) that include sequences upstream of the P2 promoter had indicated that another transcriptional start may exist within the URR. We decided to test for promoter activity in the URR with an in vitro transcription assay. A 1.5-kb fragment that includes the URR was subcloned, and the plasmid was assayed for transcriptional activity in a HeLa cell extract. Runoff transcripts were generated by digestion of the template with restriction enzymes. Digestion of the template with Hpa I gives rise to a 770-bp transcript, which would correspond to a transcriptional start approximately around nucleotide 7175 (Fig. 1A, left). This runoff product is not observed when the in vitro reaction is carried out in the presence of α-amanitin (4 μg/ml), indicating that the transcript is the product of RNA polymerase II. The start site was confirmed by generation of shorter runoff transcripts by digestion of the plasmid with Cla I and Bgl I, which cut upstream of Hpa I. To substantiate the accuracy of our in vitro system for BPV transcription, we generated runoff transcripts from the known promoter P2 by digestion of the vector with Bam HI or Eco RI (Fig. 1). The size of these runoffs, 405 and 425 bp, respectively, corresponds well with a cap site at nucleotide 89, which occurs in vivo (*11, 12*). To more accurately determine the novel point of initiation, we synthesized an oligonucleotide complementary to the sequence between 7284 and 7305 and used that for primer extension on in vitro transcribed RNA. The size of the extension product (P1) places the in vitro start at nucleotide 7186 (Fig. 1B). Interestingly, this places the cap site of the promoter within the sequences defined as necessary for ori function.

The P1 promoter was further characterized for sequences required for transcription in vitro. Deletions removing sequences up to 45 nucleotides upstream of the cap site had little or no effect on transcription, but deletions downstream of the cap site did have measurable effect (Fig. 2). In vitro transcribed RNA from different supercoiled templates was subjected to primer extension analysis with the same primer that was used previously. As an internal standard, a pseudo wild-type template was included in each transcription reaction. This template has a linker inserted at nucleotide 7212, between the sequence complementary to the primer and the cap site. Therefore, the extension product is correspondingly 16 nucleotides longer than that for the wild-type template. Primer extension analysis on RNA transcribed in vitro from three different templates with deletions downstream of the cap site (Fig. 2) shows that a deletion between nucleotide 7187 and 7234 (D234) (*10*), which removes 48 nucleotides downstream

of the cap site, completely abolishes transcriptional initiation from the P1 cap site. Two additional mutants were generated for further analysis of the regulatory element defined by the D234 mutant. Mutant D20 lacks only the 3′ 20 nucleotides of the D234 deletion, while mutant D28 lacks the 28 nucleotides just proximal to the cap site (Fig. 2B). The mutant D20 shows transcriptional activity similar to that of the wild type as measured by the ratio between pseudo wild-type and mutant extension products, while the mutant D28 lacks detectable transcriptional activity altogether. The faint band that can be seen in the D28 lane is a background band that is particularly strong in this lane. These results establish a distinctive feature of the P1 promoter; sequences downstream of the cap site are required for transcription in vitro, and the important sequences are located in a 28-nucleotide region immediately 3′ to the cap site.

The cis-acting DNA sequences required for transcription are binding sites for proteins which presumably interact with RNA polymerase (*14*). In order to establish whether the deleted sequences in the D28 deletion corresponded to such a target site for a DNA binding protein, we performed a deoxyribonuclease (DNase) footprint analysis of the region around the P1 cap site (*15*). A fragment labeled at nucleotide 7028 was mixed together with a heparin-agarose fractionated HeLa cell extract and treated with DNase to assay for protected regions. A protected region downstream of the cap site corresponds almost exactly to the sequences deleted in the D28 deletion (Fig. 2B). The footprint extends three nucleotides into the sequences deleted in the D20 deletion, which may account for why the transcriptional activity of the D20 mutant is slightly lower than wild type.

To determine whether the in vitro results described above were also relevant for transcription in vivo, we linked the DNA fragments containing the mapped cap site to the chloramphenicol acetyltransferase (CAT) gene. The constructs were assayed for expression in vivo by transfection into mouse cells and subsequent quantitation of enzyme activity in cell extracts. When the CAT coding sequence was joined to a 316-nucleotide BPV fragment at a position 90 nucleotides downstream of the P1 cap site, the fragment had promoter activity (Fig. 3). The activity of the fragment was greater in untreated C127 cells than in their BPV-transformed counterpart ID13 cells. To correct for differences in transfection efficiency between the two cell lines, expression of RSV-CAT (RSV, Rous sarcoma virus), transfected in parallel, was measured and used to normalize the values since transfection efficiencies are consistently higher for the transformed cells. The activity of the promoter was greater in the absence of BPV gene products, indicating that the P1 promoter may be negatively regulated by BPV encoded or induced factors.

When the CAT coding sequence was joined to the BPV sequence at a position one nucleotide downstream of the cap (the 5′ end of the D234 deletion was used to insert CAT), less than 0.1% acetylation of the substrate resulted. This level of acetylation is comparable to those obtained after transfection with CAT constructs that lack promoters. Our experiments leave little doubt that the internal element defined in vitro is a promoter element. If the active elements of the P1 promoter were located entirely 5′ to the cap site, we would expect significant levels of CAT activity from the D234 CAT construct.

Having established the location of the in vitro start and that the promoter was active in vivo, we determined what transcripts were generated from it. We observed earlier that no cytoplasmic RNA could be detected on Northern blots with nick-translated probes from the URR, and we tried to increase the sensitivity by treating the transformed cells with cycloheximide prior to harvest. In other viral systems, cycloheximide increases steady-state levels of viral RNA (*16*). This is true also for BPV; inhibiting protein synthesis 4 hours before harvest increases the yield of BPV messenger RNA (mRNA) 10- to 50-fold, depending on transcription unit (*17, 18*). Also, since very small exons of spliced mRNA might escape detection in a Northern analysis, we analyzed nuclear RNA, expecting that a true precursor RNA would offer a greater area of hybridization and therefore give a stronger signal. Two major bands in nuclear RNA hybridizing to the three different probes used were observed (Fig. 4). The lower molecular weight band, 4.2 kb long, corresponds to a previously characterized nuclear RNA which originates at the P2 promoter and extends to the common poly(A) site at 4203 (*11*). This RNA initiates downstream of the URR probe and consequently hybridizes only to probes A and C, which are

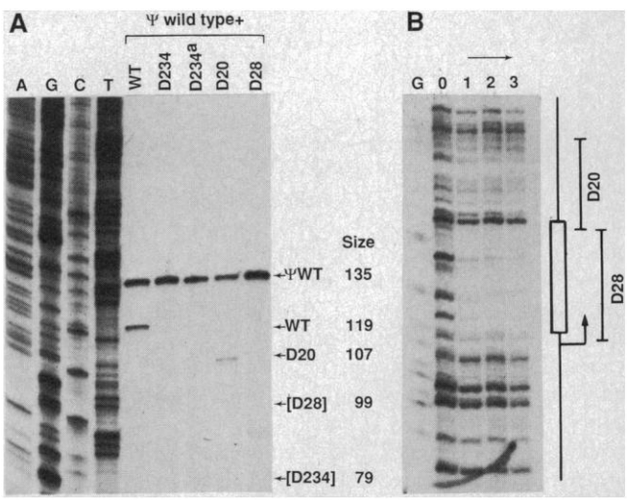

Fig. 2. (**A**) Primer extension analysis on RNA-transcribed in vitro templates with deletions downstream of the site of initiation. The respective supercoiled templates were mixed together with a pseudo wild-type template and transcribed in vitro. The resulting RNA was used as template for primer extension analysis, and the extension products for each mutant and pseudo wild-type pair were analyzed on a sequencing gel. In separate quantitative experiments, the pseudo wild-type templates were equivalent in strength to within 10 to 20%. Here, the pseudo wild type was used as an internal control, and its concentration was not adjusted to be identical to wild type. Arrows indicate where the primer extension products for each template is expected to run, together with the expected sizes in nucleotides. The mutant templates that did not give rise to detectable products are in parenthesis. The sequence ladder on the left was generated as described in Fig. 1, and serves as a size marker. (**B**) The DNase protection assay of the P1 promoter region. A DNA fragment containing the promoter region was 5′ labeled at nucleotide 7028, mixed with a HeLa cell extract fractionated on heparin agarose, and subjected to limited digestion with DNase. In lane 0, no extract was added; in lanes 1, 2, and 3, either 3, 5, or 7 μl of extract was added, respectively. Lane G is a sequence marker, generated by Maxam and Gilbert sequencing (*35*) of the same fragment that was used for the protection assay. To the right of the gel, the extent of the protected region is shown by an open bar. The relation of the protected region to the deletions D28 and D20 is indicated, and the site of transcriptional initiation is shown by an arrow. In vitro transcription and primer extension were carried out as described (Fig. 1). The wild-type template consisted of a BPV fragment extending between the Xba I at nucleotide 6132 and the Sma I site at nucleotide 945, cloned into the polylinker of pUC18; the pseudo wild-type template was identical to the wild-type template, except for the insertion of two Bam linkers (16 nucleotides) at nucleotide 7213; the mutant D234 was identical to the wild-type template except that the sequence between nucleotide 7186 and 7234 was replaced with a Bam linker; D234[a] is identical to D234, except that pML was used as a vector rather than pUC18; the mutant D28 was identical to the wild-type template except that the BPV sequences between nucleotide 7213 and 7234 were replaced by a Bam linker; the mutant D20 lacked the BPV sequences between nucleotide 7187 and 7213, which were replaced by a Bam linker. Also, the vector used in this case was pML rather than pUC18. The DNase footprint experiments were done as described (*36*) except that heparin agarose fraction h.3 (*33*) was used.

located in the P2 transcription unit. The larger band, 5.0 kb, hybridizes also to the URR probe (B) and has sequences upstream of the P2 promoter. The size of this transcript corresponds well with a colinear RNA starting around 7200 and extending to the common poly(A) site. Additional evidence that this nuclear RNA initiates at the P1 promoter at 7186 was obtained by primer extension on nuclear RNA with the same primer that was used previously. The extension product for in vivo nuclear RNA (Fig. 1B) has exactly the same size as for in vitro RNA, which means that the same start site is used. Primer extension on cytoplasmic RNA gave rise to a very faint, not readily detectable band, possibly because the sequence complementary to the primer is spliced out of some cytoplasmic RNA species.

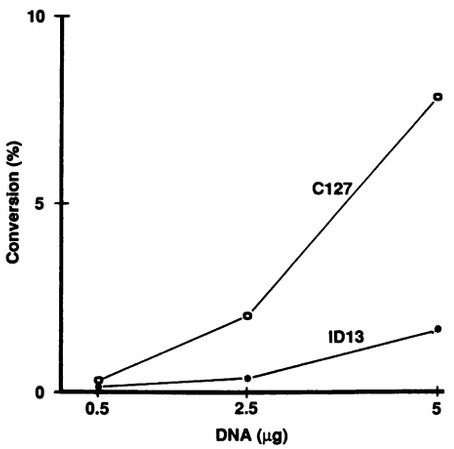

Fig. 3. Comparison of P1 promoter activity in C127 and ID13 cells. A construct with the P1 promoter linked to the CAT gene was transfected onto C127 and ID13 cells at three different DNA concentrations. The CAT activity was measured 48 hours after transfection and plotted as a function of the quantity of transfected DNA per 50-mm plate. To correct for differences in transfection efficiency between the two cell lines, the numbers were normalized to the expression of RSV-CAT (*37*) in both cell types in transfections carried out in parallel. Extracts from cells transfected with a promoterless CAT plasmid yielded less than 0.1% conversion. The normalization factor used was 1.9, meaning that the expression of RSV-CAT in ID13 cells was 1.9-fold higher than in C127 cells. This experiment was repeated several times with essentially the same result. The P1 CAT plasmid was constructed by inserting an Hind III–Nar I fragment from nucleotide 6958 to 7274 immediately upstream of the CAT coding sequence in the promoterless plasmid CAT 3M (*38*). Transfections of C127 and ID13 cells were performed as described (*39*), and cells were harvested 48 hours after the transfection. Assays for CAT activity were carried out as described (*40*) except for a few modifications. Cells were lysed by repeated cycles of freezing and thawing, and the protein concentration in the lysate was determined (*41*). The reactions contained 0.125*M* tris HCl, *p*H 7.8, 1 m*M* acetyl coenzyme A, 0.25 μCi of [^{14}C]chloramphenicol, and 75 μg of protein from the cell extracts, and were incubated overnight at 37°C.

Northern analysis of cytoplasmic RNA showed no detectable hybridization to the URR when nick-translated probes were used, even after treatment with cycloheximide; however, when high specific activity SP6 probes were used, two major bands, 1.4 and 1.8 kb, hybridizing to a small URR probe (Fig. 4A, lane D, +CH) were observed. This probe spans the origin of replication and the start site for the P1 promoter. This technique offered the sensitivity sufficient to detect these RNA's, even in cells not treated with cycloheximide (Fig. 4, lane D). Because the P1 start is linked to the origin of replication we wanted to know if the major 1.4- and 1.8-kb RNA's detected by the "D probe" were linked to the ORF E1. As mentioned above, although genetic evidence indicates that this ORF must be involved in regulating viral DNA replication, no cytoplasmic RNA containing this region has been reported. Hybridization to two probes from the E1 region (E and F) in addition to a URR probe (D) was observed (Fig. 4). The probe E, from the 3′ end of the E1 ORF, shows the presence of two bands that correspond exactly in size to the RNA's detected with the URR probe. The probe F, from the 5′ end of the E1 ORF, shows a more complex pattern with several additional bands above 1.8 kb. The high molecular weight band visible in both lanes E and F corresponds to the 4.2-kb nuclear RNA from the P2 promoter and its presence in cytoplasmic RNA is probably due to nuclear leakage. The 5.0-kb precursor is less abundant, especially in cells not treated with cycloheximide, and is not visible on this exposure. From these results we conclude that the P1 promoter gives rise to at least two stable cytoplasmic transcripts that include sequences from the E1 ORF.

The P1 promoter has a very unusual structure. Both in vitro and in vivo the

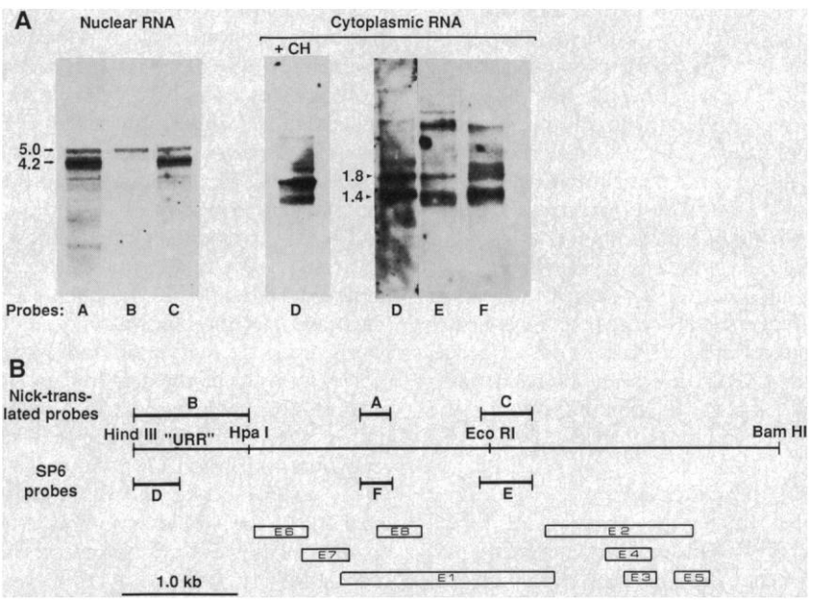

Fig. 4. (**A**) Northern blot analysis of transcripts from the P1 promoter. Strips of filters hybridized to the probes are designated by the letter below each strip; the location of these probes on the BPV genome is shown in the right panel. The three strips to the left were cut from the same gel, and each contain poly(A)-selected nuclear RNA from approximately 2×10^7 ID13 cells that were treated with cycloheximide before harvest. The three strips to the right were cut from one gel, and each contain poly(A)-selected cytoplasmic RNA from 2×10^7 ID13 cells that were not treated with cycloheximide. The middle lane contains cytoplasmic RNA from ID13 cells that were treated with cycloheximide. (**B**) Part of the BPV genome, complete with the location of the eight early open reading frames and the URR region. The nick-translated probes (A, B, and C) consist of BPV fragments subcloned into the polylinker of pUC18. The SP6 probes (D, E, and F) consist of BPV fragments subcloned into the polylinker of the SP 64/65 vectors. BPV sequences in the different probes were for probe A and F: 945 to 1299, probe B: 6958 to 93, probes C and E: 1987 to 2446, probe D: 6958 to 7352. Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cycloheximide was added to the medium at 35 μg/ml 4 hours prior to harvest. Nuclear RNA was extracted and poly(A)-selected (*42*). Cytoplasmic RNA was prepared as described (*43*). Formaldehyde-formamide denatured RNA was fractionated in 1% agarose gels containing 2.2*M* formaldehyde (*44*). After the blotting to nitrocellulose, hybridization was carried out in a solution containing 50% formamide, 50 m*M* Hepes (*p*H 7.4), 5× Denhardt's solution, yeast RNA (250 μg/ml), 3× SCC, single-stranded salmon sperm DNA (100 μg/ml), 0.1% SDS, and 5% dextran sulfate. Nick-translated probes were hybridized at 42°C, and RNA probes at 60°C. The filters were washed in 2× SSC, 0.1% SDS at 68°C. Filters probed with RNA probes were then washed again in 0.2× SSC, containing 0.1% SDS (68°C). DNA probes were labeled by nick-translation. RNA probes were generated as described (*45*).

promoter seems to have an absolute requirement for sequences just downstream of the cap site. In contrast, other transcriptional elements that have been found within a transcription unit, such as the immunoglobulin heavy chain enhancer, augments a basal level promoter and are not readily assayed in vitro (*19*). It may seem paradoxical that a transcription factor would bind downstream of the site of initiation since the transcription complex would have to pass through this region. It is, however, well established that for polymerase III transcription, binding of promoter factors to the internal promoter element is an obligatory requirement for transcription both in vivo and in vitro (*20*). The internal promoter element of P1 is defined by small deletions (Fig. 5). The 28-nucleotide sequence defined as necessary for promoter function coincides with a binding site for a factor present in a transcriptionally active extract, and also shows homology to two previously defined transcriptional elements. The sequence motif CCACACCC is an upstream domain of the β-globin promoter (*21*) and that sequence is perfectly conserved within the P1 promoter element (Fig. 5). Furthermore, the homology between this −80 domain of the β-globin element, a distal BPV enhancer motif, and an SV40 enhancer domain has been noted previously (*22*). The downstream element also contains a representative of a motif $ACCN_6GGT$, which is repeated ten times within the URR of BPV, and also occurs at high frequency in other papillomaviruses (*23*). Interestingly, a subset of this sequence, $ACCGN_4CGGT$, has been shown to form at least part of the binding site for the viral trans-acting enhancer factor E2 (*24*).

The P1 promoter is located in domain 2 of PMS1, which is the core origin of replication for BPV. This replication element, previously defined by linker insertion mutagenesis, extends from nucleotide 7116 to 7234 (*10*). The P1 promoter is completely included within this region; the sequences required for full activity in vitro extend between nucleotide 7142 and 7213. Furthermore, one specific deletion between nucleotides 7187 and 7234 (D234), which is negative for replication as reported (*10*), also abolishes transcription from P1, indicating that, in addition to the physical overlap, some sequences might be involved in both transcription and replication. One possibility is that the factor binding to the D28 region, which we believe is required for transcription from P1, also doubles as a factor required for replication. A similar situation exists in the adenovirus system, in that nuclear factor 1, NFI, which is required for replication of adenovirus DNA, is identical to the transcription factor CTF (*25*). In this latter case, though, no transcriptional function appears to be associated with the factor at the adenovirus origin.

The novel class of transcripts originating from the P1 promoter includes sequences from the 3′ and 5′ ends of the E1 ORF. Therefore we find it reasonable to believe that the replication factors M and R that are encoded from the E1 ORF are provided by the P1 promoter. This conclusion is supported by the finding that a mutant that lacks P1 promoter activity is also unable to complement either R or M mutants (*26*), suggesting that the promoter mutant is unable to produce these factors.

The location and function of the P1 promoter points to an involvement of the promoter in regulation of replication. The promoter-ori arrangement in BPV is reminiscent of the organization in SV40 and polyoma virus, where the replication factor (T antigen) is transcribed from the early promoter adjacent to the origin of replication. This arrangement, where the promoter for the positive replication factor is located very close to the origin of replication, is, curiously enough, copied in BPV, which otherwise has a completely different genome organization. This appears especially significant since in BPV there are no apparent constraints imposed on the localization of the promoter. The P1 promoter is located almost 1 kb upstream of the first major open reading frame (Fig. 4). In SV40 and polyoma virus, one reason for the physical association between origin of replication and early promoters seems to be the dual function of the positive replication factor, which has to act both as a replication factor and regulator of its own transcription (*27*). Autoregulation of the positive replication factor may also exist in BPV, as would be indicated by the down regulation of the P1 promoter that we observe in ID13 cells.

An important question is what precise function the transcriptional components have for BPV replication per se, and why an enhancer and the sequence at the promoter are required for replication even when all trans-acting factors are provided by other means. Our favored explanation for the enhancer requirement is that the enhancer element serves the same or a similar function for replication as it would for transcription, namely, to assure the proper association of transcription factors with the promoter. Therefore, if replication itself requires a transcription factor at the P1 promoter, the enhancer could be part of the system that supplies this factor. The Epstein-Barr virus (EBV) EBNA protein, which is required for EBV plasmid replication (*28*), also can act as a transcriptional enhancer factor if its binding sites are placed in cis to a heterologous promoter (*29*). It is possible that the function of EBNA in replication, as well as in transcription, is to serve as an enhancer factor that interacts directly in, or provides other factors that can function for, both replication and transcription. The requirement for a cis-acting enhancer for BPV replication has specificity. The domain 1 of PMS1 can be replaced by enhancers from polyomavirus and MLV (murine leukemia virus) (*10*), but the E2-induced enhancer (*13*) that is also located in the URR cannot serve in this function (*10*). The compatibility of an enhancer with the origin may be determined by the factor requirement of the origin of replication and the ability of the enhancer to supply these specific factors.

Several plausible functions for transcription factors at the ori can be envisioned. (i) RNA synthesis is required for initiation of DNA replication; (ii) the transcription factors serve a nonspecific function where binding of the factor facilitates a chromatin structure that is compatible with initiation of replication; and (iii) the replication ma-

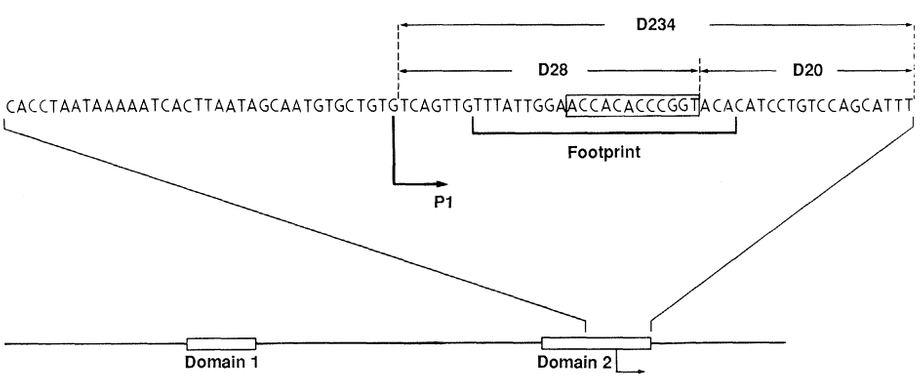

Fig. 5. A schematic representation of the PMS1 region. The lower part shows the two domains of PMS1, the upper part is an enlargement of the sequence around the P1 cap site. The cap site is indicated by an arrow, the region protected from DNase digestion is bracketed, and the deletions used are shown above the sequence. The boxed sequence is a representative of a motif, $ACCN_6GGT$, which is repeated ten times in the URR of BPV and also occurs at high frequency in other papilloma viruses.

chinery interacts specifically with a factor or factors bound to the transcriptional elements. All of these possibilities are compatible with our findings, but we believe that some aspect of point (ii) or (iii) is more likely. For SV40, polyomavirus, and adenovirus priming at the initiation step does not require RNA polymerase II, but is achieved by either a special virus encoded or cellular primase-polymerase. It is more difficult to distinguish between the functions (ii) and (iii), since both specific and nonspecific functions for DNA replication could be carried out by factors that have specificity in terms of DNA binding.

Association of transcriptional elements with origins of replication is a common observation. It is difficult to conclude whether this association is a general feature of origins, or if it reflects specific regulatory requirements for the respective replication systems, or a combination of both. An example that indicates that this association is not only a feature of viral regulation is the chorion genes of *Drosophila*. It has been shown that the chorion gene promoter resides within the origin of amplification for the chorion genes (*30*) and, even though this may represent a special case, it is possible that transcriptional elements are a general feature of cellular origins of replication as well. The link between enhancers, promoters, and replication origins in viruses may point to a method to regulate replication more directly than simply through transcriptional regulation of levels of trans-acting factors. If an intrinsic part of replication origins is a transcription factor binding site, the activity or state of this factor could directly influence the rate of replication initiation at the origin. In this way, a cycle of modifications (or other events) could serve to coordinately regulate both replication and transcription.

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Retroviruses and Mouse Embryos: A Rapid Model for Neurovirulence and Transplacental Antiviral Therapy

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A murine model in which neurotropic retroviral infection can be studied over short periods of time was developed. Microinjection of Cas-Br-E virus into midgestation mouse embryos caused paralysis and death within 25 days after birth, in contrast to virus-infected neonates which develop disease only after 4 months. To evaluate whether antiviral drugs could cross the placental barrier and influence the course of the disease, the drug 3′-azido-3′-deoxythymidine (AZT) was administered to infected embryos through the drinking water of pregnant females. AZT treatment markedly retarded the onset and course of virus-induced central nervous system disease, permitting animals to survive beyond 4 months of age. These results are evidence for effective antiviral treatment during gestation and in the perinatal period and are of potential significance for the management of maternal transmission of the acquired immune deficiency syndrome (AIDS) virus.

BOTH THE PROGRESSIVE NEUROLOGical syndromes caused by human immunodeficiency virus [HTLV-III/LAV/HIV (*1*)] and the increasing frequency of acquired immune deficiency syndrome (AIDS) in the perinatal period (*2*) are serious complications of AIDS virus infection. No short-term animal model systems exist in which to study retroviral neurovirulence and treatment strategies for neonatal and in utero infections. Here we establish a murine model system that allows the rapid study of both the interaction of retroviruses with the developing embryo and virus-induced diseases of the central nervous system (CNS). We demonstrate that transplacental antiviral therapy with 3′-azido-3′-deoxythymidine (AZT) (*3*) is an effective treatment for rapidly fatal CNS disease following virus infection of embryos in utero.

The murine neurotropic type C retroviruses Cas-Br-E (isolated from a wild mouse population) and temperature-sensitive variants of Moloney murine leukemia virus (Mo-MuLV) induce progressive lower mo-

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