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Long-Term Potentiation in Dentate Gyrus: Induction by Asynchronous Volleys in Separate Afferents

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Long-term potentiation (LTP), a long-lasting enhancement of synaptic efficacy, is considered a model for learning and memory. In anesthetized rats, activation of dentate granule cells by stimulating either the medial or lateral perforant pathway at frequencies of 100 to 400 Hz produced LTP of the stimulated pathway preferentially at 400 Hz. However, hippocampal pathways do not normally fire at this high rate. Stimuli at 200 Hz were then applied to either the medial or lateral pathway separately, to both pathways simultaneously, or to the two pathways asynchronously so that the composite stimulus applied to the granule cell dendrite was 400 Hz. LTP was produced preferentially in the asynchronous condition. Thus, lower frequency, physiological input volleys arriving asynchronously at medial and lateral synapses can induce LTP by activating a 400-Hz sensitive mechanism capable of integrating spatially separated granule cell inputs. This may reflect how LTP is normally produced in the dentate gyrus.

LONG-TERM POTENTIATION (LTP) of synaptic efficacy in the hippocampal formation occurs in dentate gyrus granule cells after repetitive stimulation of the medial perforant path. Trains of stimuli applied at 10 to 100 Hz for 2 to 20 seconds produce increases in efficacy that persist for hours in anesthetized preparations and for days to weeks in freely moving animals (1). LTP is most reliably elicited by stimuli applied at 400 Hz (2); trains of pulses at this frequency are currently utilized for the induction of LTP in this structure (3).

The physiological relevance of such stimuli depends on whether such trains of action potentials with interspike intervals of 2.5 msec are normally propagated in the hippocampal formation. Pyramidal cells of the CA1 and CA3 fields do fire in rapid, short bursts of 2 to 7 action potentials termed complex spikes (4). Such bursts might be capable of inducing LTP in their target areas in the hippocampal formation. However, interspike intervals of 2.5 msec or less occur infrequently (5). The firing rates of the cells of the entorhinal cortex which, via the medial and lateral perforant pathways, constitute the input to the dentate gyrus are unknown. However, the granule cells themselves do not exhibit complex spikes and fire at a rate

of less than 100 Hz (6). Thus, physiological firing of hippocampal pathways as currently known does not generally reach 400 Hz. In our study we have quantified the relative efficacy of 400-Hz stimulation of the dentate gyrus in inducing LTP, and ascertained how this high frequency effect might be realized in normal animals by asynchronous inputs of lower frequency acting upon separate regions of the granule cell dendrite.

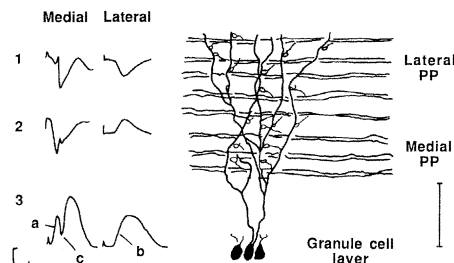
In urethane-anesthetized rats, a recording electrode was lowered to the granule cell layer of the dentate gyrus and stimulating electrodes were positioned to activate both the medial and lateral perforant pathways. The medial and lateral perforant pathways are distinct pathways that originate in different subfields of the entorhinal cortex (7) and innervate separate regions of the granule cell dendritic tree (Fig. 1). The two pathways may be clearly differentiated physiologically by their middle third field responses (Fig. 1, left). When we recorded in the middle third region of the dendrites (position 2), a single pulse applied to the medial perforant path resulted in a negative potential at a latency of approximately 2 msec. This is termed the evoked synaptic potential (ESP) and is a measure of the flow of current into the dendrite as a result of synaptic activity. At

position 3, the positive potential (designated "a") reflects the corresponding outward flow of current from the cell body. A sufficiently high intensity of stimulation of the medial pathway elicits a population spike ("c"), which constitutes a measure of the number of granule cells firing action potentials. Stimulation of the lateral perforant path elicits an ESP in the outer region of the dendrites (position 1) at a somewhat longer latency (see below). The corresponding ESP at the cell layer is designated "b." Lateral path stimulation, even at saturation intensities, fails to elicit a population spike. A distinguishing feature of the comparative field responses is that at the level of maximum negative ESP of the medial pathway (position 2), the lateral ESP has reversed to a positivity. In these experiments, as in previous studies in the dentate gyrus (8), the slope of the ESP at the cell body layer is utilized as a measure of synaptic efficacy.

In all experiments it was first verified that the medial and lateral perforant paths were being stimulated separately (9). We recorded in the granule cell layer to determine input-output curves for the slope of the ESP for both the medial and lateral pathways and, in some cases, the amplitude of the population spike of the medial response (Fig. 2B, baseline). The effect of the frequency of stimulation on the induction of LTP was determined by testing either the medial or the lateral pathway in a given rat. In each animal a perforant path (PP) current for tetanic stimulation (LTP current) was selected slightly above that required to elicit a threshold response. The objective was to choose a current sufficiently low such that LTP would not be induced at any frequency between 100 and 400 Hz. A test current, approximately two-thirds of the saturation current, was chosen to test for the induction of LTP. The test current (a single 250- μ sec pulse) was applied at 1-minute intervals for 10 minutes to establish a baseline response, and for 20 minutes after each application of

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Fig. 1. (Right) Fibers of the lateral and medial perforant pathways (PP) are shown making en passage synaptic contact with dendrites of dentate gyrus granule cells. The lateral and medial pathways have their cells of origin in the lateral and medial entorhinal cortex, respectively (to the right of the illustration but not shown), and, respectively, synapse with the outer and middle third of the granule cell dendritic tree. (Left) Stimulation of the lateral path results in a maximum negative ESP at position 1, while stimulation of the medial path elicits a maximum ESP at position 2. At position 2, the lateral ESP has already reversed to a positivity. In the granule cell layer a population spike (c) can be elicited by stimulation of the medial pathway but not the lateral. See text for further details. Calibrations 4.5 mV and 4.0 msec, positivity upward. Vertical bar on right, 100 μ m.



LTP stimulation. Once substantial LTP had been induced, test pulses were applied for 30 minutes or more, and a final input-output curve was determined. LTP stimulation consisted of five trains of pulses separated by 1 second. Each train consisted of five pulses applied successively at 100, 200, 300, and 400 Hz, with intervening test periods for the evaluation of LTP induction (as noted) interposed between each frequency of application (10). If LTP was not induced at any frequency, the LTP current was increased by 50 to 75 μ A and the procedure

repeated. Current levels were raised until LTP was induced.

Data were obtained from eight animals—four were used to test the medial pathway and four for the lateral. Two representative experiments are shown in Fig. 2A. In the case of the medial pathway, LTP was not produced at a current of 250 μ A. At 320 μ A, there was a 33% increase in the slope of the ESP after 400 Hz stimulation while there was no increase at lower frequencies. In the case of the lateral pathway, LTP was not induced at 150 μ A. At 200 μ A, succes-

sively higher frequency trains produced small increases of the ESP slope up to 400 Hz compared to the immediately preceding responses. These were 8% at 100 Hz, 2% at 200 Hz, and 4% at 300 Hz. At 400 Hz, however, there was a considerably larger (17%) increase. Results in the other six rats were similar. Either no LTP was produced until 400 Hz (two lateral, two medial; the range of mean increase at 400 Hz was 22 to 36%) or the increase at 400 Hz was at least twice the increase at any lower frequency (one medial, one lateral). Taking the medial group as a whole, increases of the slope at 400 Hz compared to the immediately preceding responses were significant [$32.5\% \pm 2.3$ (SEM), t test, $df = 3$, $P < 0.001$], as was the case in the lateral group ($19.7\% \pm 1.1$, t test, $df = 3$, $P < 0.001$). In each individual animal, the increase of slope after LTP was significantly greater than baseline at all PP current levels (two-way analysis of variance, $P < 0.001$) (see Fig. 2B).

We also tested whether lower frequency inputs arriving asynchronously on medial and lateral pathways (so that the composite input applied to the granule cell dendrite was 400 Hz) would similarly produce LTP preferentially. LTP stimuli were a series of five trains of five pulses each as described above (Fig. 3, top). However, in this experiment, the frequency was uniformly at 200 Hz (5-msec interpulse interval). LTP trains were applied first to one pathway and then to the other; each LTP train was followed by a 20-minute test period. (The two pathways were tested alternately at 30-second intervals.) After LTP application to the individual pathways, trains were applied to both pathways simultaneously. In all cases, the fiber volley generated by the incoming action potentials was utilized to assure synchrony. Synchronous LTP application was followed by a test period and a final application of LTP trains with one volley delayed with respect to the other so that the composite interpulse interval was 2.5 msec (400 Hz) (11). As in the first experiment, LTP currents were initially set near threshold values so that LTP was not induced during any of the stimulus conditions. LTP current was then increased until substantial LTP was produced.

Data were obtained from seven rats. A typical experiment is shown in Fig. 3A. The asynchronous volley alone produced substantial LTP. There was a 16% rise of the ESP slope in the medial pathway and a 24% rise in the lateral pathway, as compared to the preceding test period. Small degrees of LTP were induced by either single or synchronous application of LTP trains, but the asynchronous volleys produced LTP at least three times any preceding condition. Results

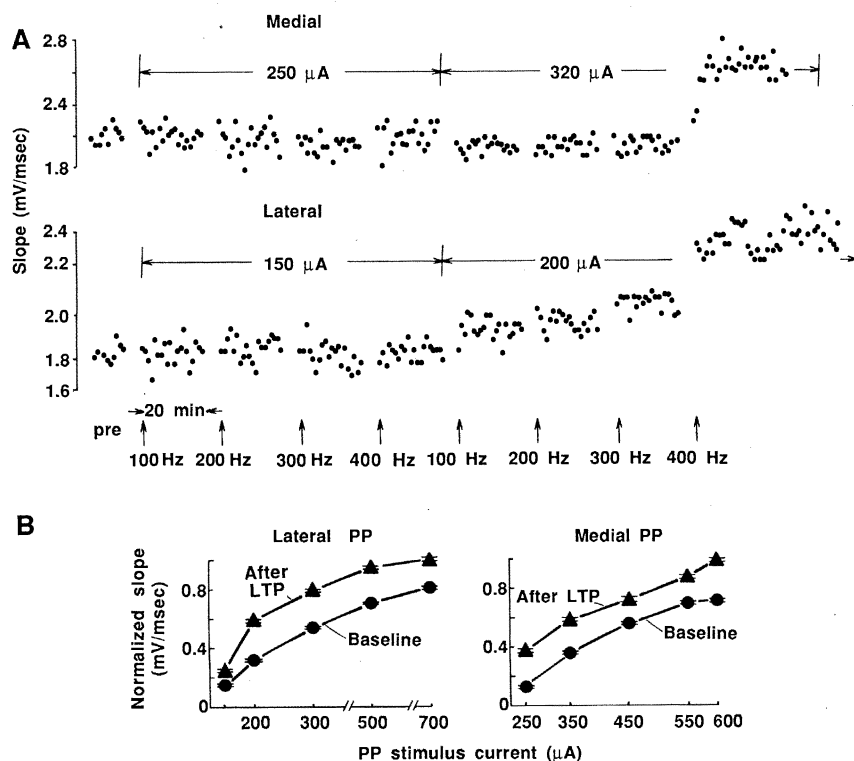


Fig. 2. (A) Representative examples of medial and lateral perforant paths (PP) tested separately in two rats. Starting at intensities of tetanic perforant-path stimuli below the threshold for inducing LTP, LTP stimulation was applied successively at 100, 200, 300, and 400 Hz. LTP current was then raised until LTP was induced. In all experiments, this occurred preferentially at 400 Hz (see text for further details). In the examples shown, the perforant path current utilized to test the ESP of evoked responses was 300 μ A on the lateral path and 450 μ A on the medial. (B) Input-output curves were determined before the application of tetanic stimuli (baseline) and at the conclusion of the experiment by measuring 15 responses at each stimulating current intensity (after LTP). Responses were taken at 15-second intervals. Horizontal bars are standard errors of the mean. Responses were normalized to the maximum ESP. Maximum lateral ESP, 6.7 mV/msec; maximum medial ESP, 7.0 mV/msec.

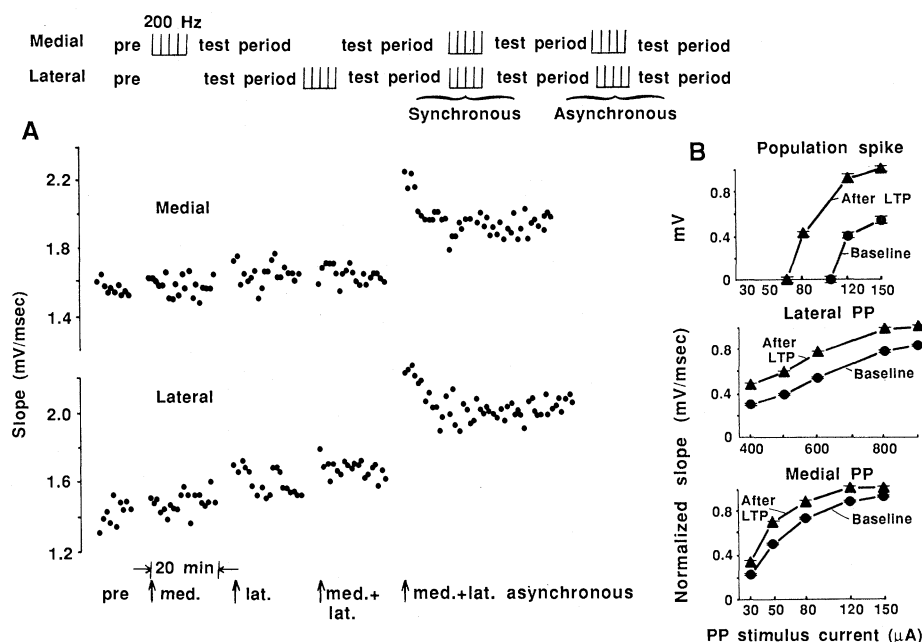


Fig. 3. (A) LTP stimuli were applied to both lateral and medial pathways and the pathways were tested for the induction of LTP. LTP stimuli were applied at 200 Hz either separately to each pathway, simultaneously to both, or asynchronously so that the composite stimulus was at 400 Hz. The test paradigm is illustrated at the top of the figure. LTP was induced preferentially following asynchronous stimulation. Shown are the LTP currents at which the LTP was first produced. The test current was 80 μ A in the medial pathway and 600 μ A in the lateral pathway in this example. In this animal, as in most, the asynchronous volleys produced initial posttetanic potentiation that decayed over a period of 5 to 8 minutes (17). (B) Input-output curves revealed significant LTP at all perforant path (PP) current levels in both the ESP's and the medial population spike. At the test current of 80 μ A, there was no population spike before asynchronous stimulation and a substantial spike afterwards (upper plot). Responses were normalized to the maximum ESP. Maximum population spike, 18.2 mV; maximum lateral ESP, 10.6 mV/msec; maximum medial ESP, 12.3 mV/msec.

were similar in the six other rats (ranges of increase: medial, 14 to 26%; lateral, 16 to 32%; increases were at least 2.5 times larger than any immediately preceding LTP effect). For the seven rats as a group, the increases in the asynchronous condition were significant ($21.7\% \pm 2.3$, t test, $df = 6$, $P < 0.001$). In each individual animal the asynchronous volleys produced significant ($P < 0.001$) increases in the ESP (Fig. 3B, lower two plots) as well as the population spike (Fig. 3B, upper plot) at all perforant path current levels.

Finally, we used a modification of the same paradigm; a single burst of five pulses at 200 Hz (rather than five bursts at 1-second intervals) was applied singly, synchronously, and asynchronously to induce LTP. Initial LTP currents were set at higher levels (approximately 60% saturation for the lateral pathway and PP currents sufficient to produce a small population spike in the medial response). Data were obtained from three rats. The asynchronous volleys induced substantial and significant increases in the ESP (average $18.3\% \pm 2.5$, t test, $df = 2$, $P < 0.005$) and the other stimulus conditions produced no change in the ESP or increases less than one-third of that obtained following the asynchronous volleys.

Both neuropsychological findings and the

results of animal experiments suggest that the hippocampus plays an essential role in memory (12). A three-stage anatomical substrate for such a role may exist. There is a known convergence to entorhinal cortex of neocortical inputs from various sensory modalities, a major projection (perforant path) from the entorhinal cortex to the hippocampus (primarily to the dentate gyrus), and a return projection from the hippocampus to the entorhinal cortex via the CA1 field (13). In conjunction with activation of this circuitry, LTP induced in hippocampal circuitry via the perforant path during the occurrence of an event may provide synaptic facilitation enabling its recall (14). Our findings may be pertinent to this formulation as follows. Medial and lateral perforant path projections to the dentate gyrus appear to transmit information derived preferentially from different sensory modalities (15). Near simultaneous but asynchronous volleys propagated along these pathways at physiological frequencies during the occurrence of an event may induce LTP of selective synapses on granule cell dendrites by triggering a 400-Hz-sensitive mechanism (16). This may form the basis in the normal animal for later association of information in the various modalities that constituted the event, allowing for its recall.

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9. The criteria utilized were appearance of the maximum lateral ESP approximately 100 μ m dorsal to the maximum medial ESP, reversal of the lateral ESP at the dorsoventral level of the maximum medial ESP, elicitation of a population spike in the medial but not the lateral response at the granule cell level and the longer latency of the lateral versus medial fiber volley and response as described in (11).
10. Responses and numerical values of ESP slope were monitored on line on a computer display. Additional input-output curves were established within a run when it was judged that small but consistent increases in the ESP followed tetanic stimulation.
11. The precise synchrony or 2.5-msec asynchrony of medial and lateral inputs was made possible by the appearance of a clear fiber volley in recordings at the granule cell layer preceding the evoked response. The latency of the fiber volley after lateral perforant path stimulation was approximately 1 msec longer than the medial (with a consequent later evoked response). Synchrony was obtained and verified in each experiment by delaying medial perforant path stimulation compared to the lateral by the increased latency of the lateral volley. Asynchrony was obtained by delaying the medial stimulation an additional 2.5 msec or, in alternate experiments, delaying the lateral stimulation beyond its normal approximately 1 msec longer latency so that the lateral volley lagged behind the medial by 2.5 msec.
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16. It is suggested that in the normal animal lower frequency volleys, perhaps of the order of 100 Hz or less (the reported firing rate of granule cells), arriving asynchronously on a number of perforant path fibers may constitute the composite 400-Hz input produced here by asynchronous 200-Hz volleys. (In the normal animal the asynchrony could be paced by internal circuitry within the entorhinal cortex or could result from the asynchrony inherent in diverse neocortical input to the entorhinal cortex.) The 400-Hz composite volley could then activate the 400-Hz-sensitive mechanism demonstrated here to produce LTP in selected synapses.
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18. Supported by NIMH Research Scientist Development Award 5-K02-MH00232, NSF grant BNS 8209875, and a grant from the Harry F. Guggenheim Foundation to J.W.

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Expression and Characterization of the *Trans*-Activator of HTLV-III/LAV Virus

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The human T-lymphotropic retrovirus HTLV-III/LAV encodes a *trans*-activator that increases viral gene expression. We expressed this *trans*-activator in animal cells and studied its structural and functional characteristics. The putative *trans*-activator protein was immunoprecipitated from overproducing stable cell lines and shown to migrate as a 14-kilodalton polypeptide on sodium dodecyl sulfate-polyacrylamide gels. S1 nuclease mapping experiments showed that the *trans*-activator increases the levels of steady-state messenger RNA transcribed from the viral long terminal repeat promoter. Sequences within the R region of the HTLV-III/LAV long terminal repeat are essential for *trans*-activation. Quantitations of messenger RNA and protein showed that the protein increase was greater than the messenger RNA increase in CV1 and HeLa cells, indicating that more than one mechanism was responsible for the *trans*-activation and that cell type-specific factors may determine the final level of *trans*-activation.

THE HUMAN RETROVIRUS HTLV-III/LAV [or human immunodeficiency virus (HIV) (1)] is the etiologic agent of acquired immune deficiency syndrome (AIDS) (2-4). Elucidation of the molecular structure of the virus (5) revealed that it is related to lentiviruses, such as visna virus (6). In addition to the three genomic retroviral regions *gag*, *pol*, and *env*, all the AIDS virus isolates contain additional reading frames that encode proteins of mostly unknown function. One genomic region necessary for the activation of the HTLV-III/LAV long terminal repeat (LTR) has been identified. Complementary DNA (cDNA) clones and deletion mutants of the virus were used in cotransfection experiments to identify the genomic region that encodes the *trans*-activator (7, 8). The genomic region was called the *tat*-III gene and was proposed to encode a transcriptional *trans*-activator of the HTLV-III/LAV virus (8). Within this genomic region, an open reading frame (ORF) is conserved in all of the viral isolates that have been sequenced to date. Cotransfections of cell lines with plasmids containing this intact ORF for the putative *trans*-activator protein increased the amounts of chloramphenicol acetyltransferase (CAT) produced from the LTR pro-

motor in transient expression assays (7, 8). Substantial evidence indicates that the protein product of this conserved reading frame is the *trans*-activator of HTLV-III/LAV. The cDNA clones and deletion mutants that destroy the reading frame are negative, whereas all the constructs that contain the conserved ORF are positive in *trans*-activation assays (7, 8). We have identified the protein product of the *tat*-III ORF. Since the mode of action of this protein is not yet clear, we refer to this putative *trans*-activator as TA-III. Here we demonstrate that the *trans*-activator increases the steady-state level of messenger RNA (mRNA) transcribed from the viral LTR promoter as well as the level of the produced protein. Quantitative comparisons indicate that the level of protein increases more than can be accounted for by the mRNA levels.

Three types of vectors were used for the expression of the *trans*-activator of HTLV-III/LAV in animal cells (Fig. 1). A bovine papilloma virus (BPV) vector was constructed containing a Sal I-Xho I fragment from either of two HTLV-III/LAV proviral clones [pHXB2C or pBH10R (9, 10)] next to the mouse metallothionein-I (mMT-I) promoter. This construct (pB2MX3) was introduced into mouse C127 cells by the

calcium coprecipitation technique (11). Two days later the cells were placed in medium containing 10 μ M CdCl₂ to select for cells resistant to cadmium because of overproduction of human metallothionein (hMT) encoded by the vector. Cadmium-resistant colonies were cloned and maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 20 μ M CdCl₂. Independent cadmium-resistant cell lines were assayed for the presence of the HTLV-III/LAV *trans*-activator. A convenient functional assay (Fig. 2A) was used to screen 22 cell lines for the presence of functional *trans*-activator expressed from the mMT-I promoter. Cells were transfected with plasmid pL3CAT (Fig. 1B), which contains the LTR promoter of HTLV-III/LAV that transcribes the bacterial CAT gene (12). CAT assays on 2 of the 22 cell lines (CB2MX3-23 and CB2MX3-24) are shown in Fig. 2A. All 22 cell lines selected on the basis of cadmium resistance stimulated the production of CAT at levels 40 to 300 times those of cell lines that contained only the BPV vector. Clones CB2MX3-2, CB2MX3-23, and CB2MX3-24 were selected for further analysis. The induction of CAT production in these cell lines compared with control CBMG7-4 cells were 300-fold, 100-fold, and 300-fold, respectively.

To identify the expressed protein, we labeled CB2MX3-2 cells with [³⁵S]cysteine for 1 hour, lysed them in RIPA buffer (Fig. 3), and the cellular extracts were immunoprecipitated with sera from AIDS patients or a rabbit antiserum to the *tat*-III ORF expressed in bacteria (13) (Fig. 3). The putative *trans*-activator protein was immunoprecipitated as a 14-kilodalton (kD) protein by the rabbit antiserum. The discrepancy between the predicted molecular weight of 10 kD (7) and the observed migration on SDS gels could be caused either by post-translational modifications or by anomalous migration due to the basic nature of this protein. Sera from three AIDS patients used in the same assay were not able to immunoprecipitate the 14-kD protein under the same conditions. However, other studies have shown that sera from some AIDS patients recognize this 14-kD protein (13, 14). A higher molecular weight band of 26 kD was also present in some immunoprecipitations.

The BPV construct as well as expression vectors based on simian virus 40 (SV40) (Fig. 1) were also used for the transient

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