maize mutants. They both suffer from a deficiency of endogenous GA_1, and their poor shoot growth can be increased through the exogenous application of GA_3.

Thus, inbreeding depression in maize is due, in part, to a partial GA deficiency. Conversely, heterotic hybrids contain higher concentrations of GA_1 and hence their shoot growth is more vigorous. This model indicates that GAs play a major regulatory role in heterosis for maize shoot growth. Since shoot growth is positively correlated with grain yield (*15*), this model also implies a role for GAs in the regulation of heterosis for the economically important character of grain yield.

The enhanced production of GA_1 in heterotic hybrids is probably the result of an enhancement of overall GA biosynthesis in the hybrids. Rates of GA_{20} and GA_1 metabolism, including glucosyl conjugation and 2β hydroxylation, are probably faster in at least one maize hybrid than in its parental inbreds (*2, 16*). Hence, to bring about increased GA_1 levels, biosynthetic rates must be very much faster in the hybrids. This enhanced biosynthesis could result from enzymic polymorphism, which is known to occur for a number of enzymes in maize and has been proposed as a general molecular explanation for heterosis (*17*). This model suggests that the existence of different isozymes allows the hybrid to perform better under a broader range of environments and developmental stages. Consequently, the heterozygous condition of the hybrid confers biosynthetic superiority.

Possibly as a result of enzymic polymorphism, GA concentration is enhanced and this enhanced GA level, in turn, induces enhanced biosynthesis of numerous other compounds; GAs are known to play a regulatory role in the induction of gene expression in higher plants (*7*). Thus, amplification of just a few genes responsible for GA biosynthesis could accelerate or activate numerous subsequent biosynthetic pathways. The consequent metabolic cascade could thereby amplify the advantages of the heterozygote. Although this specific mode of action is speculative, our results confirm that GAs are important in the regulation of heterosis, or conversely, inbreeding depression, in maize. Maize hybrids display hybrid vigor, at least in part, as a result of an enhanced GA concentration while the less productive parental inbreds are limited by a partial GA deficiency.

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Effect of Neuropeptides on Production of Inflammatory Cytokines by Human Monocytes

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Two groups of mediators, the neuropeptides substance P and K and the monocyte-derived cytokines, interact in the neural regulation of immunological and inflammatory responses. Substance P, substance K, and the carboxyl-terminal peptide SP(4–11) induce the release of interleukin-1, tumor necrosis factor–α, and interleukin-6 from human blood monocytes. The neuropeptide effects occur at low doses, are specific as shown by inhibition studies with a substance P antagonist, and require de novo protein synthesis. Since monocyte-derived cytokines regulate multiple cellular functions in inflammation and immunity and since neuropeptides can be released from peripheral nerve endings into surrounding tissues, these findings identify a potent mechanism for nervous system regulation of host defense responses.

THE CONCEPT THAT THE NERVOUS system modulates immunological and inflammatory responses has been supported by the identification of neuropeptide receptors on leukocytes and the demonstration that these peptides can regulate leukocyte functions (*1*). Mononuclear phagocytes, either as circulating blood monocytes or as tissue macrophages, influence host defense responses through their capacity to present antigens and to release several types of soluble mediators (*2*). These monocyte-derived cytokines act in a paracrine fashion in the local environment to stimulate immune responses, but also in an endocrine-like fashion on distant organs that participate in inflammatory responses. Systemic changes during inflammation such as increased acute-phase protein synthesis and fever probably represent action of these cytokines on liver and hypothalamus. Interleukin-1 (IL-1) and tumor necrosis factor–α (TNF-α) were the first monocyte-derived cytokines shown to have diverse regulatory properties in immunity and inflammation (*3, 4*).

Recently, a third molecule, termed B cell stimulatory factor–2 (BSF-2), interferon-β2, 26-kD protein, hybridoma-plasmacytoma growth factor, now known as interleukin-6 (IL-6), augments the activation of T lymphocytes, triggers acute-phase protein synthesis in hepatocytes, and probably induces febrile responses (*5–8*).

We examined the ability of substance P (SP) and related neuropeptides to regulate the production of inflammatory cytokines by human blood monocytes. We showed that the neuropeptides SP and substance K (SK) are potent and specific stimuli for the production of IL-1, TNF-α, and IL-6.

This interaction of neuropeptides that are released from unmyelinated sensory neurons in response to traumatic or inflammatory stimuli (*9*) with monokines that mediate localized and systemic host defense responses constitutes a potent mechanism for neural regulation of immunity and inflammation.

Blood monocytes cultured in the presence of SP released increased levels of IL-1 measured as thymocyte stimulatory activity. The effects of SP were detectable at doses as low

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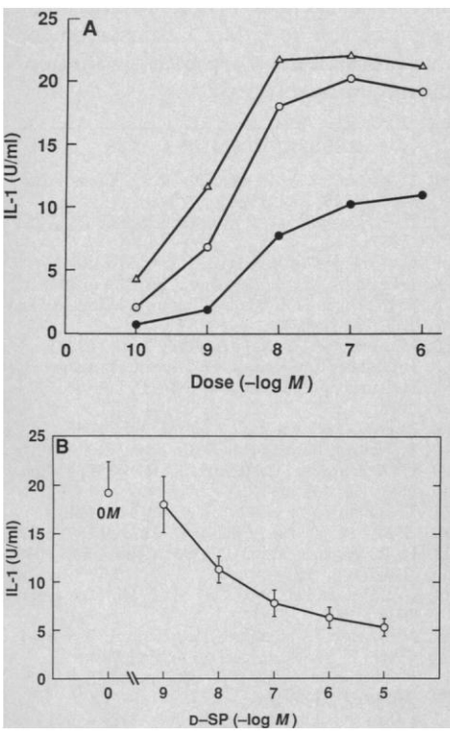
as $10^{-10}M$ and were maximal between 10^{-8} and $10^{-7}M$ (Fig. 1A). The truncated COOH-terminal peptide SP(4–11) and SK also gave a similar dose-dependent release of IL-1. The neuropeptides by themselves did not have detectable thymocyte stimulatory activity. Comparison with another monocyte activator, lipopolysaccharide, showed that the maximal levels of IL-1 induced by SP ($10^{-8}M$) corresponded to 38.4% of the IL-1 activity in monocyte cultures stimulated with lipopolysaccharide (100 ng/ml). Kinetic analyses showed that, in neuropeptide-supplemented monocyte cultures, increased IL-1 production was detectable by 6 hours and continued to increase for another 30 hours. The supernatant from monocytes cultured in medium containing serum and polymixin B (12.5 μg/ml) alone displayed minimal thymocyte stimulatory activity. Since monocytes release at least two cytokines that can augment thymocyte proliferation, namely, IL-1 and IL-6, we characterized the activity in the neuropeptide-stimulated cultures. Preincubation of monocyte culture supernatants with antibody to human IL-1β (*10*) significantly reduced the thymocyte stimulatory activity (Table 1). Between 30 and 50% of the activity resisted treatment with antibody to IL-1. However, antibody to human BSF-2/IL-6 (*11*) also diminished the thymocyte stimulatory activity in the monocyte supernatants, whereas immunoglobulin G (IgG) from unimmunized rabbits had no effect. These results indicated that stimulation of monocytes with the neuropeptides SP or SK induced release of both IL-1 and IL-6. To confirm this observation, we tested the monocyte supernatants using two specific bioassays for IL-6. In the CESS cell assay, IL-6 increases IgG secretion (*12*), while in the T1165 plasmacytoma assay, IL-6 specifically enhances growth (*13*). As tested by both assays, high levels of IL-6 activity were detected in culture supernatants from neuropeptide-stimulated monocytes. Neuropeptides SP and SK at 10^{-8} to $10^{-7}M$ induced maximal IL-6 activity (Fig. 2). The activity was specific and it was neutralized by antibody to BSF-2/IL-6 (Table 1). Experiments with highly purified monocytes (antibody-complement lysis with antibodies to B and T lymphocytes) showed very similar levels of SP-induced IL-1, suggesting that small numbers of lymphocytes do not detectably contribute to the SP effects on monocytes.

Subsequent experiments were performed to establish the specificity of the SP effect. For this purpose we used an SP antagonist that has substitutions with D-isomers of several amino acids. The antagonist, D-Pro^2,

Table 1. Neutralization of IL-1 and IL-6 activity. Monocytes (>95% nonspecific esterase–positive) were isolated from peripheral blood of healthy donors as described (*20*) and cultured in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, Massachusetts) at 10^6 cells per milliliter in RPMI 1640, supplemented with 5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 m*M* L-glutamine (all from M.A. Bioproducts, Los Angeles, California) and polymixin B (12.5 μg/ml) (Burroughs Wellcome, Research Triangle Park, North Carolina). Culture supernatants from monocytes stimulated with SP and SK (Peninsula Laboratories, Belmont, California) were collected at 36 hours and tested in the mouse thymocyte (*21*) and the T1165 assay (*13*). Proliferation was measured by [^{3}H]thymidine uptake at 72 and 48 hours, respectively. For neutralization studies, portions of the supernatants were preincubated for 2 to 4 hours at 37°C in the presence of monospecific antibody to IL-6 (rabbit IgG fraction) diluted 1:10 in medium, equivalent concentrations (10 μg/ml) of preimmune rabbit IgG (*11*), or antibody to human IL-1 (*10*). Data are shown as counts per minute ± SEM of six determinations. ND, not determined.

Condition	Mouse thymocyte assay		T1165 assay	
	$10^{-8}M$ SP	$10^{-8}M$ SK	$10^{-8}M$ SP	$10^{-8}M$ SK
No antiserum	12,644 ± 932	18,342 ± 1,632	9,426 ± 876	12,934 ± 1,394
Anti–IL-1 1:100	4,685 ± 672	3,924 ± 586	ND	ND
Anti–IL-1 1:500	6,726 ± 609	9,439 ± 1,072	ND	ND
Anti–IL-6 10 μg/ml	7,293 ± 911	10,392 ± 1,219	876 ± 172	1,311 ± 302
Anti–IL-6 1 μg/ml	10,302 ± 1,192	14,768 ± 1,738	3,296 ± 674	4,387 ± 506
Control 10 μg/ml	11,932 ± 1,326	19,422 ± 1,832	9,942 ± 1,072	13,862 ± 1,042
Serum 1 μg/ml	13,439 ± 1,044	20,396 ± 1,659	10,752 ± 1,396	14,012 ± 1,230



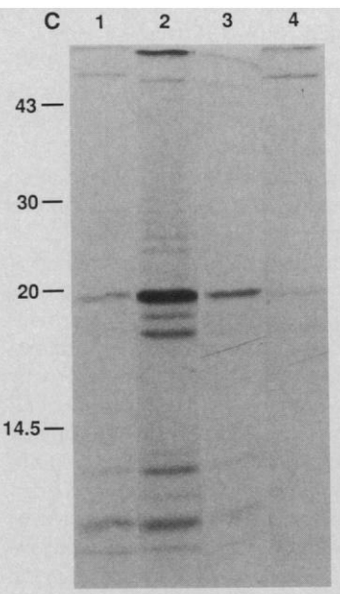


Fig. 1. (**A**) Purified blood monocytes were stimulated with the indicated doses of SP (○), SK (△), and SP(4–11) (●) for 36 hours. Culture supernatants were tested in the mouse thymocyte assay where proliferation was measured after 72 hours. One unit of IL-1 was defined as the amount of IL-1 that induces a twofold increase in thymocyte proliferation over phytohemagglutinin background. Data are from three representative experiments performed in triplicate. (**B**) Monocytes were stimulated with ($10^{-8}M$) SP and varying doses of the antagonist D-SP. Supernatants were collected and tested as described above. Results are from two representative experiments. Error bars are SEM. (**C**) SP induces de novo synthesis of IL-1. Purified monocytes were cultured at 10^6 cells per well in 24-well cluster plates in methionine-free medium supplemented with [^{35}S]methionine (1 μCi/ml). Culture supernatants were collected at 68 hours, clarified by centrifugation at 1600*g*, and passed through 0.45-μm filters. The filtrates were cleared with 10 μl of 10% protein A in NET buffer [50 m*M* tris-HCl (*p*H 7.4), 0.5% NP-40, 0.15*M* NaCl, 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 5 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (20 μg/ml), 5 μ*M* leupeptin, and 5 μ*M* antipain]. Immune adsorption of the IL-1 in culture supernatants was performed by rotating 200 μl of spent medium with 40 μl of a 1:1 suspension of protein A Sepharose and rabbit antiserum for 1.5 hours at 4°C. The Sepharose beads were washed ten times in NET buffer to remove the nonadsorbed proteins. The proteins were released from the beads by boiling in sample buffer (0.0625*M* tris, *p*H 6.8, 3% SDS, and 10% glycerol containing 0.025% bromphenol blue) and separated on 12.5% polyacrylamide slab gels. The gels were dried and developed by autoradiography. Lane 1, control; lane 2, $10^{-8}M$ SP; lane 3, $10^{-8}M$ SP and $10^{-5}M$ D-SP; and lane 4, normal rabbit serum.

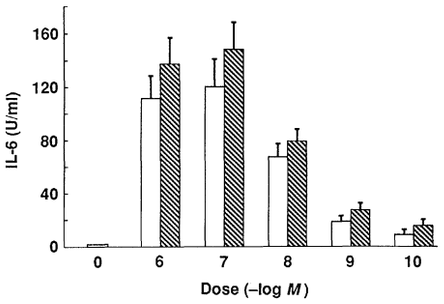


Fig. 2. Culture supernatants (36 hours) from monocytes stimulated with SP (open bars) or SK (hatched bars) were tested in the CESS assay for IL-6 as described (*12*). Briefly, 5×10^3 CESS cells in each well of a 96-well plate were incubated in the presence of test samples for 5 days. Supernatants were then harvested, and total IgG production was quantitated by means of an enzyme-linked immunoassay (ELISA). Results are expressed in units of IL-6 activity, based on a standard curve derived from recombinant IL-6. All samples were tested in triplicate for each experiment.

Table 2. Induction of TNF activity by neuropeptides. TNF activity was measured with the standard cytotoxicity assay with the use of actinomycin D–treated L929 cells. Briefly, 30,000 cells per well were cultured in 96-well flat-bottomed tissue culture plates in the presence of test samples and actinomycin D (1 μg/ml). After 18 hours of incubation, cell lysis was determined by staining with crystal violet, and dye uptake was measured in an ELISA reader. A standard was included in each assay by using recombinant human TNF-α. One unit of TNF is the amount that induces 50% lysis of the L929 target cells. Monocyte culture supernatants collected at 36 hours were tested at several dilutions in the L929 assay. In neutralization studies, supernatants were preincubated (2 hours at 37°C) with antibody to TNF-α or lymphotoxin (LT) (donated by H. M. Shepard, Genentech) and tested for residual activity. Data are shown as units of TNF activity and represent means ± SEM of three experiments, each done in triplicate. ND, not determined.

Stimulus	Total TNF activity (U/ml)	Residual activity after treatment with antibody	
		To TNF-α	To LT
None	9 ± 3	<4	6 ± 3
$10^{-7}M$ SP	424 ± 31	38 ± 7	392 ± 26
$10^{-8}M$ SP	538 ± 28	34 ± 9	548 ± 42
$10^{-9}M$ SP	226 ± 17	15 ± 9	192 ± 20
$10^{-10}M$ SP	47 ± 7	<4	38 ± 8
$10^{-8}M$ SP(4–11)	392 ± 19	ND	ND
$10^{-8}M$ SP + $10^{-6}M$ D-SP	62 ± 11	ND	ND
$10^{-6}M$ D-SP	12 ± 5	ND	ND
$10^{-8}M$ SK	468 ± 33	53 ± 13	432 ± 48

D-Phe^2, D-Trp^9-SP, reduced in a dose-dependent manner the levels of IL-1 that had been induced by $10^{-8}M$ SP (Fig. 1B and Table 1). Similarly, SP ($10^{-8}M$) induced 140 ± 36 U of IL-6 per milliliter, which was reduced to 24 ± 12 U/ml by the simultaneous presence of D-SP ($10^{-6}M$). The antagonist itself did not stimulate cytokine release from monocytes and did not interfere with lipopolysaccharide-induced production of IL-1. These results and the fact that polymixin B was included in all cultures to inhibit endotoxin effects supported the concept that the induction of IL-1 and IL-6 from monocytes was a specific function of the neuropeptides.

Next, we wished to determine whether the SP-induced increase in IL-1 release was related to de novo protein synthesis. In these experiments monocytes were incubated for 36 hours in methionine-free RPMI supplemented with [^{35}S]methionine at 1 μCi/ml. Then the culture supernatants were immunoprecipitated with antiserum to IL-1. In the absence of neuropeptides, cultured monocytes released minor amounts of metabolically labeled IL-1. The presence of SP ($10^{-8}M$) caused a clear increase in the protein band below 20 kD, which corresponds to the molecular size of secreted human IL-1β. This appeared to be a relatively selective effect, since the quantity of other proteins nonspecifically precipitated by the antiserum did not significantly change (Fig. 1C). These experiments also showed that the SP antagonist inhibited SP-induced synthesis of IL-1.

Finally we examined the monocyte cultures for the production of TNF-α, the third inflammatory monokine. Monocytes treated with SP released high levels of material with cytolytic activity (Table 2). The maximum SP effect occurred at $10^{-8}M$. Substance K and truncated SP(4–11) were slightly less potent. The SP-induced increase in TNF activity was inhibited by the antagonist D-SP. Neutralization studies with antibodies to TNF-α and lymphotoxin showed that most of the cytolytic activity was due to TNF-α, a finding that is consistent with this cytokine being a monocyte product.

These findings show how immunological and inflammatory responses are regulated by nervous system–derived signals. We demonstrate that the neuropeptides SP and SK induce release of IL-1, IL-6, and TNF-α by human blood monocytes. The spectrum of SP effects on monocytes is probably not limited to the induction of cytokine production. Other monocyte functions regulated by this neuropeptide include activation of arachidonic acid metabolism, chemotaxis, and oxidative burst (*19*). IL-1 and TNF-α are critical in the initiation of humoral and cellular immune responses and stimulate systemic changes during inflammation, including synthesis of acute-phase proteins and the induction of fever. The monokines may also induce connective tissue destruction in chronic inflammatory diseases (*3, 4*). IL-6 has recently been identified as a mediator with pleiotropic effects. It was earlier known as BSF-2, which induces differentiation of B lymphocytes into antibody-secreting cells, and also referred to as interferon-β2 (IFN-β2), 26-kD protein, or hybridoma-plasmacytoma growth factor (*5*). IL-6 is now also known to regulate growth of hemopoietic stem cells (*14*), fibroblasts (*15*), and T lymphocytes (*6, 16*), and to induce acute-phase proteins (*7*) and possibly fever (*8*). In multiple different cell types, including endothelial cells, hepatocytes (*17*), and fibroblasts (*15*), IL-6 can be induced by IL-1 and TNF-α, which may represent an important amplification mechanism for host defense responses.

In an earlier study we demonstrated that SP stimulates production of prostaglandin E_2 and collagenase by rheumatoid synoviocytes (*18*). Our findings that SP and SK augment the release of IL-1, TNF-α, and IL-6 provide evidence for the potentially pivotal role of neuropeptides in the pathogenesis of a wide range of inflammatory diseases. It is also likely that SP may stimulate release of IL-1 or IL-6 in other SP-responsive tissues, such as fibroblasts and endothelial cells, and the effects on monocytes are consistent with the expression of SP receptors on these cells (*19*).

The significance of the present in vitro studies is supported by the antidromic release of SP from peripheral nerve terminals into surrounding tissues (*9*), and the detection of increased SP levels in inflamed tissues of patients (*1*). The results suggest that specific antagonists of the neuropeptides might be able to reduce inflammation and thus provide a novel approach toward treatment of many diseases.

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A Novel Gene of HIV-1, *vpu*, and Its 16-Kilodalton Product

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A 16-kilodalton protein expressed in cells producing the human immunodeficiency virus (HIV-1) was identified as the gene product of the *vpu* open reading frame. When expressed in vitro, the 81–amino acid *vpu* protein reacted with about one-third of the serum samples from AIDS patients that were tested, indicating that the *vpu* open reading frame is expressed in vivo as well. Introduction of a frame-shift mutation into the *vpu* open reading frame did not significantly interfere with expression of the major viral proteins in a transient expression system. However, a five- to tenfold reduction in progeny virions was observed after the infection of T lymphocytes with the mutant virus. These data suggest that the *vpu* gene product is required for efficient virus replication and may have a role in assembly or maturation of progeny virions.

LIKE OTHER MEMBERS OF THE LENtivirus subfamily, HIV-1 contains several open reading frames (ORFs) in addition to the *gag*, *pol*, and *env* ORFs present in other retroviruses. Some of these genes, such as *tat*, *rev* (formerly *art/trs*), and *nef* (*3'-orf*, B), are thought to regulate viral gene expression. Another ORF, *vpr* (R), has been reported to be expressed in vivo even though replication of HIV-1 in T lymphocyte cell lines is not affected by the presence or absence of a functional *vpr* gene (*1*). In this study, we show that another small (81 codons) HIV-1 ORF, designated *vpu* ["U," see (*2*)] encodes a 16-kD protein that is expressed in vitro and in vivo. Mutagenesis of *vpu* in an infectious molecular clone of HIV-1 results in the synthesis of particles with altered replicative capacity.

The *vpu* gene product was initially studied by translating, in vitro, SP6-directed RNA transcripts of subcloned segments of HIV-1 proviral DNA. Two plasmids were constructed for that purpose (Fig. 1A). The first, pSP-6, could potentially encode the first exons of *tat* (72 amino acids) and *rev* (27 amino acids), the initial 756 codons of the *env* gene, and the 81-residue *vpu* gene product. The second, pSP-9 could specify only the *vpu* protein and a truncated (292–amino acid) *env* protein. The coding capacities of both plasmids can be further reduced by restriction enzyme digestion prior to in vitro transcription. Linearization of both pSP-6 and pSP-9 DNAs at the Kpn I site interrupts the *env* gene at codon 42 but leaves the *tat*, *rev*, and *vpu* ORFs intact in pSP-6. Cleavage of pSP-6 DNA with Hind III eliminates *env* and *vpu* coding sequences but does not affect the first exons of *tat* and *rev*.

Run-off RNA transcripts from linearized pSP-6 and pSP-9 plasmids were synthesized and translated in rabbit reticulocyte lysates. Proteins were metabolically labeled with [^{35}S]methionine and reacted with serum from either a seronegative individual or an AIDS patient and analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Fig. 1B, pSP-6 RNA "A," which contained *tat*, *vpu*, and *env* coding sequences, elicited the synthesis of 14-, 16- and 80-kD proteins that immunoreacted with the AIDS patient serum. The shorter pSP-6 RNA "B," which has the capacity to code for *tat* and *vpu* products as well as a truncated (42-residue)

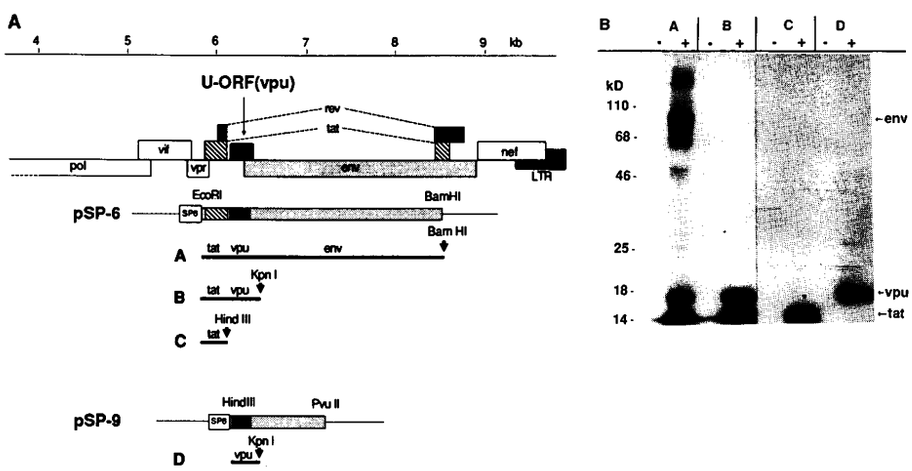

Fig. 1. Expression of *vpu* in vitro and immunoprecipitation of HIV-1–specific proteins with AIDS patient serum. (**A**) Two plasmids were constructed for the in vitro transcription of HIV-1–specific sequences: pSP-6 contains a 2.75-kb Eco RI–Bam HI fragment (nucleotides 5779 to 8522) from the infectious molecular clone pNL432 (*3*) cloned into pSP65 (*4*). Similarly, pSP-9 contains a 1.1-kb Hind III–Pvu II restriction fragment (nucleotides 6022 to 7130) cloned into the same vector. In both constructs initiation of translation of the *vpu* protein starts at internal sites: The start codons for *vpu* correspond to the third and second AUG codons on the pSP-6 and pSP-9 transcripts, respectively. pSP-6 DNA was linearized with Bam HI (A), Kpn I (B), or Hind III (C). pSP-9 DNA was linearized with Kpn I (D). Run-off transcripts (shown by solid bars) were synthesized in a reaction containing 40 m*M* tris-HCl, *p*H 7.5, 6 m*M* $MgCl_2$, 2 m*M* spermidine, 10 m*M* DTT, 1 U/μl of RNasin (Promega), 500 μ*M* rNTP's, linearized DNA (100 μg/ml), and SP6 RNA polymerase (1 U/μg DNA) for 1 hour at 40°C (*4*). Arrows show the sites used for linearization of the DNA templates. The individual genes are designated according to the new nomenclature for HIV-1 (*13*). (**B**) Aliquots of the transcripts "A" to "D" corresponding to 0.5 μg of DNA template were used for in vitro translation in a reaction containing 40 μl of rabbit reticulocyte lysate, 10 μl of [^{35}S]methionine (10 μCi), and 5 μl of RNA. The translation reaction was performed at 30°C for 1 hour. HIV-1–specific proteins were immunoprecipitated with an AIDS patient serum (+) or a negative control serum (−) and separated on a 10 to 20% SDS-polyacrylamide gel prior to autoradiography. The proteins immunoprecipitated with the AIDS patient serum are indicated on the right.

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