cosahexaenoic fatty acids (in fish) are more unsaturated (two double bonds compared to five and six double bonds, respectively), of a longer chain length (18 carbons compared to 20 and 22 carbons, respectively), and of the ω-3 rather than ω-6 type. In addition to this last point, differences in metabolism of ω-6 and ω-3 fatty acids correlate well with altered production of various prostaglandins, thromboxanes, and prostacyclins (*18*), but the exact relation of these end-products to insulin action is unknown. It may be that the combination of ω-3 fatty acids and increased chain length is crucial. Studies with linolenic acid (an 18-carbon ω-3 fatty acid found, for instance, in linseed oil) would help to clarify this issue.

Another possible mechanism involves the altered membrane fluidity that results from the substitution of fish oil in the diet (*19*). Although more rigid membranes with the same number of receptors are capable of binding more insulin (*20*), events subsequent to binding, such as aggregation, internalization of the insulin-receptor complex, and movement of the glucose transporter to the cell membrane, could all be facilitated by changes in membrane fluidity.

Finally, ω-3 fatty acids are potent inhibitors of very low density lipoprotein (VLDL) synthesis in the liver (*21*). Since VLDL-triglycerides are an important energy source in peripheral tissues, a mechanism based on fuel switching with reduced fatty acid and increased glucose utilization (the glucose–fatty acid cycle of Randle) cannot be ignored.

Care must be taken when extrapolating results from rats to humans. The amount of fat in the high-fat diet (59% of the total calories) is greater than the average intake estimated for individuals in Western societies (40% to 45%). Further, the relation of fat intake to insulin resistance in humans has been established largely on the basis of epidemiological studies, and there is little evidence concerning alterations in insulin sensitivity in humans after the kind of changes in dietary fat described here. Finally, the substitution of ω-3 fatty acids in the rat diets, while small in terms of percentage, would still represent a large intake (8 to 9 g per day) in humans. Nevertheless, therapy combining modest increases in ω-3 fatty acid intake with general reduction in total fat may be particularly effective in the dietary treatment of non–insulin-dependent diabetes mellitus.

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15. The calculation assumes that the glucose metabolic index R_g', derived from 2-DG phosphorylation, accurately estimates absolute glucose metabolic rate. There is evidence (*14*) that this assumption holds in skeletal muscle.
16. The values for R_a and R_d in Fig. 1, B and C, may be underestimates because of possible [^{14}C]glucose recycling (*13*). However, estimates based on plasma disappearance of isotopically labeled 2-DG (which overestimates R_a and R_d because of loss of tracer in urine) provide the same conclusion regarding relative diet effects. In absolute terms, if allowance is made for 15 to 20% recycling of labeled glucose at these insulin levels and for 16% loss of labeled 2-DG in urine (unpublished observations), then excellent agreement between the two calculations is achieved.
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The *sor* Gene of HIV-1 Is Required for Efficient Virus Transmission in Vitro

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The genome of the human immunodeficiency virus HIV-1 contains at least eight genes, of which three (*sor*, *R*, and 3′*orf*) have no known function. In this study, the role of the *sor* gene was examined by constructing a series of proviral genomes of HIV-1 that either lacked the coding sequences for *sor* or contained point mutations in *sor*. Analysis of four such mutants revealed that although each clone could generate morphologically normal virus particles upon transfection, the mutant viruses were limited in their capacity to establish stable infection. Virus derived from transfection of Cos-1 cells (OKT4$^-$) with *sor* mutant proviral DNA's was resistant to transmission to OKT4$^+$ "susceptible" cells under cell-free conditions, and was transmitted poorly by coculture. In contrast, virus derived from clones with an intact *sor* frame was readily propagated by either approach. Normal amounts of *gag*-, *env*-, and *pol*-derived proteins were produced by all four mutants and assays in both lymphoid and nonlymphoid cells indicated that their trans-activating capacity was intact and comparable with wild type. Thus the *sor* gene, although not absolutely required in HIV virion formation, influences virus transmission in vitro and is crucial in the efficient generation of infectious virus. The data also suggest that *sor* influences virus replication at a novel, post-translational stage and that its action is independent of the regulatory genes *tat* and *trs*.

CONSIDERABLE PROGRESS HAS BEEN made in defining the genetic structure of HIV-1 and delineating the complex array of genes encoded by the 9.7-kilobase RNA molecule of this virus. To date, eight genes have been described: *gag, pol, env* (genes encoding conventional structural elements of the retrovirus), *tat*-III, *art*/*trs* (regulatory genes that are obligatory for virus replication), *sor*, *3′orf*, and *R* (of undefined function) (*1*).

The *sor* gene (for short open reading frame) of HIV-1 (also called Q, P′, orf-1, and orf-A) lies between the *pol* and *tat* genes, overlapping at its 5′ end with the former (*2*). It is an open reading frame of 609 nucleotides in size and encodes a pro-

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tein of 23 kD (*3*). The role of this gene in virus replication and pathology is unclear. It appears to be conserved among all HIV isolates (*2*) and in the distantly related simian and human viruses STLV-III^{AGM} and HIV-2 (*4*), and a comparable open reading frame is found in lentiviruses (caprine arthritis-encephalitis virus and visna) (*5*), suggesting that this gene may be functionally significant.

Initial studies with *sor* deletion mutants of HIV-1 suggested that *sor* is dispensible for virus replication and cytopathic effects (*6*). Slight reductions in the rate of virus replication with *sor* mutants were noted, and these were attributed to disturbances in neighboring *tat* and *pol* genes (created inadvertently when deleting segments from the *sor* gene). It was also reported that high levels of *tat*-III expression appeared to compensate for the *sor* defect (*6*). We have conducted parallel studies to clarify the role of *sor*, using a series of mutants of the biologically active molecular clone pHXB2-D (*7*). A deletion mutant ΔS was created by removing the sequences between the Nde I and Nco I restriction enzyme sites (see Fig. 1). This mutant is similar to the pDsor2 mutant described by Sodroski and colleagues (*6*). In addition, three variants (3.3, 6.9, and 153) were produced by site-directed mutagenesis. These mutants were designed to disrupt the *sor* reading frame (by inserting translational stop codons) without affecting *tat* and *pol*. Since there are four potential initiator methionine residues located within the first 40 amino acids of the *sor* frame, each termination codon was inserted downstream from this last methionine codon. This, we anticipated, would ensure the production of severely truncated *sor* proteins.

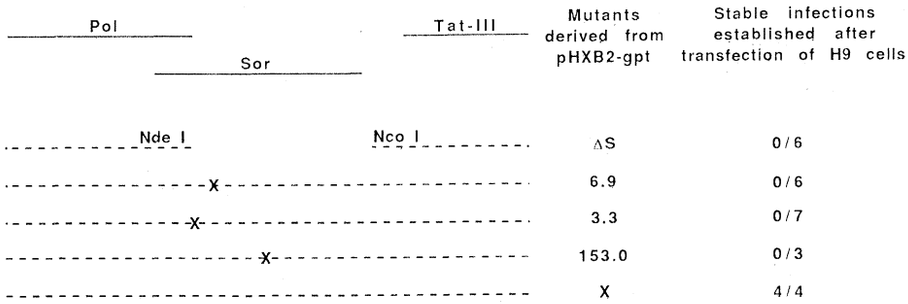

Fig. 1. Construction and properties of *sor* mutants of HIV-1. Plasmid X was generated from pHXB2gpt (*16*) by removal of an Eco RI site in the polylinker of the vector. Mutants ΔS, 3.3, 6.9, and 153 were produced from the proviral clone X in the following manner. ΔS was prepared by restriction cleavage and removal of the sequences between the Nde I and Nco I restriction sites (nucleotides 4707 to 5259) with the use of conventional methods (*17*). Site-specific mutations were prepared by oligonucleotide-directed mutagenesis. These mutations introduce translational stop codons into the *sor* frame (downstream of the *sor*/*pol* overlap) and their positions are shown diagramatically as --X--. To construct these mutants we subcloned the Eco RI to Eco RI fragment (nucleotides 4230 to 5322) of the HIV-1 genome of λBH10 (*18*) into an M13 phage vector, and performed mutagenesis as described previously (*19*). Sequences in this region of BH10 differ from those of HXB2 only at nucleotide position 4506 which results in a proline to serine substitution in *pol*. Mutation 6.9 was introduced by using the 25-nucleotide fragment (GGATGAGGGCTTTCTTAGTGATGCT), converting the tyrosine codon (TAT) at residue 55 into a stop codon (TAA). In mutation 3.3 the serine codon (TCA) at position 42 was replaced with a stop codon (TAA) by using the 24-nucleotide fragment (CCTAGCTTTCCCTTAAACATACAT). For mutation 153, a 23-nucleotide fragment (TTCCTCCATTATATGGAGACTCC) was used to change the glutamine codon (GAA) at residue 100 to a stop codon (TAA). After confirming the structures by DNA sequencing, we subcloned the mutated fragments into the proviral clone X and transfected them into H9 cells by protoplast fusion (*7*). Cultures were monitored for HIV-1 production throughout the course of each experiment (*20*).

DNA from each of the four variants was first introduced into permissive lymphoid cells (H9, Molt 4, and phytohemagglutinin-stimulated blood mononuclear leukocytes), by means of protoplast fusion (*7*). The transfected cultures were monitored at approximately weekly intervals for the appearance of HIV-1 *gag* and *env* (determined by the reactivity with antibodies to p17, p24, and gp41), reverse transcriptase (RT), and virus particles (detected by electron microscopy). As illustrated in Fig. 1, H9 cells transfected with *sor* mutants ΔS, 3.3, 6.9, and 153 consistently failed to express virus as detected by our assays. Southern blotting analyses on these samples showed transient uptake of plasmid DNA but no detectable proviral sequences in long-term cultures. Likewise, Molt 4 cells or normal T cells transfected with *sor* mutants did not establish stable virus expression, while those transfected with control genomes (X and

Table 1. Infectivity and properties of *sor* mutant viruses of HIV-1. Molt 3 cells that had been infected with HIV-1 by coculturing with transfected Cos-1 cells (as described in Fig. 3) were used as sources of X, ΔS, 3.3, 6.9, and 153 virus. Cells that had been cocultured with pSV2neo transfected Cos-1 cells were used as negative controls. Culture supernatants were removed from 1×10^8 actively growing Molt 3 cells after 5 days, concentrated 100× by centrifugation (100,000*g* for 1 hour at 4°C), and resuspended in fresh culture medium. The infectivity of these preparations was determined by mixing 100 μl of virus suspension (at various dilutions) with 3×10^5 Polybrene-treated (2 μg/ml for 30 minutes at 37°C) H9 cells (in 50 μl), incubating for 1 hour at 37°C (with mixing) and returning the samples to culture in RPMI 1640 medium containing 20% FCS (2 ml). Two weeks later the cultures were screened for HIV-1 expression (with antibody BT3). Detection of HIV-1 p24 reactive cells was determined by indirect immunofluorescence (+ indicates reactive cells, − indicates no reactive cells). Reverse transcriptase (RT) activity was determined on 100× supernatants (concentrated with polyethylene glycol) by using standard protocols (*11*). Values shown as "dpm" represent the amount of ^3H incorporated with the use of dT·rA as a primer template (the amount of ^3H incorporated when dT·dA was used as a template was in all cases <4300 dpm). The density of virus particles contained in culture supernatants was determined by negative staining of 100× concentrated samples and direct counting by electron microscopy. The results shown are from a single experiment performed in parallel. Comparable results were seen in two repeat experiments with Molt 3 and Cos-1 derived–virus.

Sample	Supernatants removed from Molt 3 cultures and concentrated ×100			Infectivity of fivefold dilutions of culture supernatants					
	RT activity		Virus concentration (particles per milliliter)	1	1:5	1:25	1:125	1:625	1:3125
	dpm	[^3H]dTMP (pmol hour^{-1} ml^{-1})							
X	138,857	36.0	3.7×10^{10}	+	+	+	+	−	−
ΔΣ	137,743	35.6	2.6×10^{10}	−	−	−	−	−	−
3.3	120,262	31.2	2.9×10^{10}	−	−	−	−	−	−
6.9	74,794	19.4	2.4×10^{10}	−	−	−	−	−	−
153	61,383	15.8	3.3×10^{10}	−	−	−	−	−	−
ΔSV2neo	2,944	<0.1		−	−	−	−	−	−

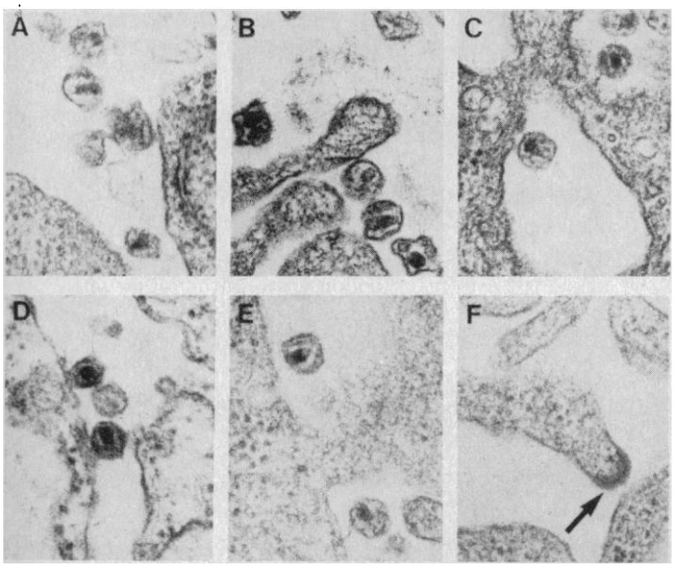

Fig. 2. Virus production by Cos-1 cells transfected with *sor* mutants of HIV-1. Electron micrographs showing virus production by SV40-transformed Cos-1 cells after transfection with plasmid clones X (full-length provirus) (**A**) and the *sor* mutants 3.3 (**B**), 6.9 (**C**), 153 (**D**), and ΔS (**E** and **F**). Extracellular, mature, immature, and particles and budding virus (F, arrow), were observed in all samples. Transfection was carried out by the calcium phosphate–mediated approach (*14*) in which 10 μg of cesium chloride gradient purified plasmid DNA was introduced into 1×10^6 Cos-1 cells. After transfection (48 to 72 hours) the cells were dislodged by using a rubber policeman, washed in phosphate-buffered saline (PBS), and fixed in 1.25% glutaraldehyde prior to processing for electron microscopy.

pHXB2gpt) reproducibly and rapidly yielded virus-producing cells. These results have several possible explanations: (i) the *sor* mutants were unable to generate virus, (ii) the *sor* mutant genomes generated virus at such low levels that it failed to establish stable infection, or (iii) virus derived from the transfected cells was defective in its ability to be propagated in culture. To distinguish among these possibilities, we performed a series of transfections using the SV40 transformed cell line Cos-1 as a target. Our aim was to amplify virus production by exploiting the capacity of the Cos-1 cells to promote episomal replication of plasmids carrying the SV40 origin of replication (including plasmids derived from pHXB2gpt) in transient assays (*8*).

As shown in Fig. 2, virus particles morphologically similar to wild type were recovered from Cos-1 cell cultures transfected with *sor* mutants 6.9, 3.3, 153, and ΔS. The level of virus production (both extracellular and budding virions) was indistinguishable from that seen in pHXB2-gpt and X transfected cultures. However, supernatants removed from *sor* mutant cultures (containing cell-free virus) did not successfully infect H9 cells in repeated attempts. These results indicate that the *sor* gene of HIV is not critical for the elaboration of normal levels of morphologically intact virus particles.

The failure of $OKT4^+$ cells to become stably or productively infected after transfection with *sor* mutated genomes (or infection with Cos-1–derived virus), even though normal levels of virus particles were made, led us to speculate that *sor* might be important in the production of infectious virions. To investigate this hypothesis, we conducted transmission studies in which Cos-1 transfected cells ($OKT4^-$ donors) were cocultured with H9 or Molt 3 cells ($OKT4^+$ targets) and the frequency of HIV-1 expressing cells among the recipient populations was monitored with time. In this way the transmissibility of the *sor* mutant viruses was compared. As shown in Fig. 3A, H9 cells that had been cocultured with either pHXB2gpt or X transfected Cos-1 cells showed a rapid increase in the proportion of HIV-1–infected cells [measured as the percentage of *gag* (p24) reactive cells] reaching >50% of the population in about 10 days. In contrast, cultures derived from *sor* mutants showed a peak incidence of only 0.4% to 1.2% at 10 days after coculture, and the incidence of virus-positive cells declined slightly over the next 3 weeks. The reduced incidence of *gag*-positive cells in cultures derived from each of the *sor* variants (ΔS, 3.3, 6.9, and 153) compared with controls (pHXB2gpt, and X) (Fig. 3) shows that removal or attenuation of *sor* leads to a reduction in the transmissibility of the resultant virus. It is interesting that HIV-1 *gag*-expressing cells persisted in the ΔS, 3.3, 6.9, and 153 cultures at low levels (0.1% to 0.4% of population) for as long as they were monitored (>70 days). Since these cultures were dividing rapidly, the absolute number of virus-expressing cells is estimated to have actually increased with time, suggesting that virus derived from the *sor* mutants was transmissible in culture, albeit inefficiently. The rare positive cells in these cultures were

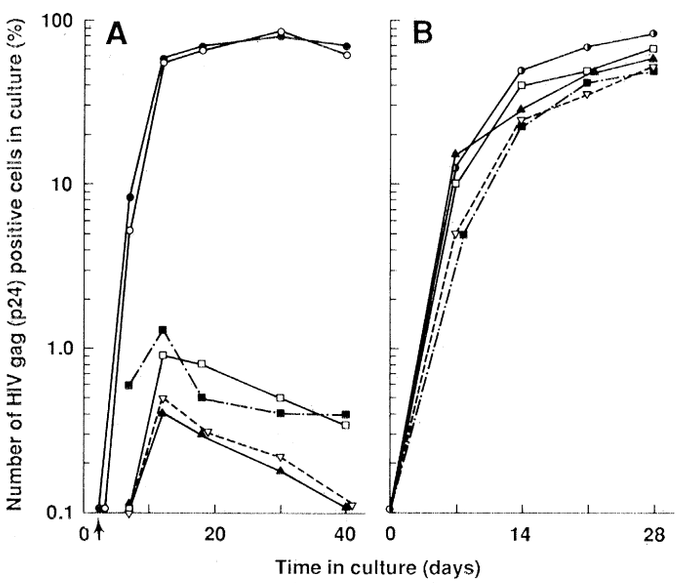

Fig. 3. Transmission of *sor* mutant viruses. Cos-1 cells were transfected with plasmid DNA from pHXB2gpt (●), X (○), ΔS (■), 3.3 (□), 6.9 (▲), and 153 (▽), as described in Fig. 2. At 6 to 24 hours after transfection, 1×10^6 to 2×10^6 Polybrene-treated (2 μg/ml for 30 minutes at 37°C) H9 cells (**A**) or Molt 3 cells (**B**) were added, and these monolayers were cocultured in Dulbecco's minimum essential medium containing 10% fetal calf serum (FCS) for a further 48 to 72 hours. The nonadherent cells resuspended in fresh RPMI 1640 medium containing 20% FCS and antibiotics. The incidence of HIV-1 *gag* expressing cells in these H9 or Molt 3 cultures was determined at regular intervals after coculture (see arrow), by means of the monoclonal antibody BT3, acetone/methanol fixation of cells, and an indirect immunofluorescence assay. For each individual determination of the percentage immunofluorescent positive cells, at least 10^5 cells were scanned. Results in (A) summarize data from three to five experiments performed independently, where the values shown for pHXB2gpt and X varied by no more than 20% between individual experiments and the values obtained for ΔS, 3.3, 6.9, and 153 had standard deviations (relative to total cells) of not more than 1.2, 0.8, 0.37, and 0.2%, respectively. Results in (B) are the averages of two experiments. Treatment of Cos-1 cells with mitomycin C (140 μg/ml for 60 minutes) prior to coculture with H9 cells gave results comparable to those shown in (A). Cultures originating from Cos-1 cells transfected with pSV2neo were used as negative controls. In these cultures the percentages of cells reactive with antibody to p24 (*gag*) were in all instances <0.01% and are therefore omitted from Fig. 3.

often found associated in small groups (of two to three cells).

In parallel experiments in which Molt 3 cells were used as coculture recipients, a different transmission profile was observed (Fig. 3B). HIV-1 infection progressed in all cultures. The rise in HIV-1–positive cells was slightly delayed in the case of *sor* defective mutants (3.3., 6.9, 153, and ΔS) as shown by the increase in time taken to achieve 50% infection (21, 22, 27, and 28 days, respectively) compared with the parental strains (14 to 15 days). These data confirm that *sor* enhances HIV transmission in vitro and indicate that enhancement may vary according to cell type. It is possible that intrinsic cellular factors, including the differentiation or activation stage of the cell, may in part determine the influence of *sor*.

The transmissibility of *sor* mutant viruses was studied in more detail by comparing the infectivity of virus derived from infected Molt 3 cells. An example of these studies is shown in Table 1. Abundant HIV-1 particles and high levels of RT activity were detected in all culture supernatants except those carrying no HIV-1 sequences (Molt 3/pSV2neo). Clarified supernatant containing wild-type virus (X) was infectious when inoculated into H9 cultures. Analysis of serial fivefold dilutions showed infectivity at concentrations of 1/5, 1/25, and 1/125. In contrast, supernatants containing *sor* mutant viruses (ΔS, 3.3, 6.9, and 153) failed to infect H9 cells at any of the dilutions tested. These data indicate that virus lacking *sor* is at least 100 times less efficient at "cell-free" infection of H9 cells.

To investigate whether the reduced infectivity of *sor* mutant virus resulted from a failure to generate normal viral RNA transcripts and proteins or to *trans*-activate genes linked to the HIV-1 long terminal repeat regions (LTR's), we performed a series of experiments. These experiments were intended to reveal (i) at what stage in the viral life cycle the *sor* gene exerts its effect and (ii) whether the reduced infectivity of *sor* mutants could be ascribed to direct effects resulting from altering the *sor* gene product or to indirect effects where *sor* modulates the expression or action of other viral genes.

Northern blotting analyses (Fig. 4) indicated that the amount and quality of viral RNA produced by Molt 3 cells infected with the parental (X, lane 1) or *sor* mutant viruses (ΔS, 6.9, 3.3, and 153; lanes 2, 3, 4, and 5, respectively) were similar. Bands corresponding to HIV-1 genomic (9.5 kb), envelope (4.5 kb), and 3′*orf, tat* RNA transcripts (2 kb) were detected in all infected cultures and were absent from uninfected controls (lane 6). Likewise, Cos-1 cells transiently transfected with DNA from the *sor* deletion mutant ΔS (lane 8) showed a pattern and abundance of viral RNA species comparable with that of the wild type (lane 7). Small discrepancies in the amounts of viral RNA were seen (less than fivefold) in multiple repeat experiments with the use of different plasmid preparations, passages of cells, transfection efficiencies, and extent of sample degradation. Detection of normal levels of viral RNA in cells transfected with *sor* mutants of HIV-1 suggests that *sor* does not exert its effects by influencing viral transcription. Furthermore, since the *tat*-III and *trs* genes of HIV-1 are themselves postulated to modulate RNA expression in infected cells (*9*), normal levels of RNA expression by *sor* mutants provide indirect evidence that the mechanism by which *sor* modulates virus propagation is not dependent on interplay between *sor* and either *tat* or *trs*.

Immunoprecipitation studies were performed to analyze HIV-1–specific protein expression by *sor* mutant genomes (Fig. 5). As shown in Fig. 5A, high levels of *env*- (gp160 and gp120) and *gag*- (p24 and $Pr55^{gag}$) derived proteins were evident in Molt 3 cells infected with *sor* mutants (3.3, lane 2; 6.9, lane 3) and wild-type viruses (X, lane 4). These bands were not seen in uninfected cells (lane 1). An additional virus-specific band was seen at approximately 39 kD. This species, detected in samples carrying *sor* defective and intact virus, most likely

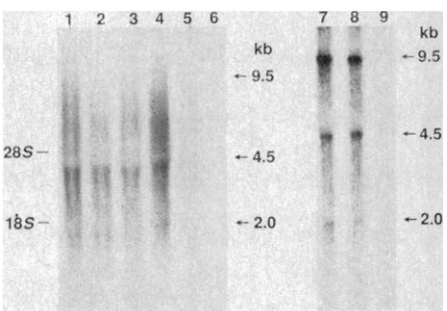

Fig. 4. Analysis of HIV-1 RNA transcripts expressed by *sor* mutant genomes. Molt 3 cultures originating from experiments shown in Fig. 3 were used as sources of X-, ΔS-, 6.9-, 3.3-, and 153-infected cell lines. Cells (1×10^7) from cultures containing >90% HIV-1 *gag* (p24)-expressing cells were washed twice with PBS, and RNA was extracted by the guanidine isothiocyanate approach (*15*). Total cellular RNA (25 μg) from Molt 3 samples infected with X, ΔS, 6.9, 3.3, and 153 virus (lanes 1, 2, 3, 4, and 5, respectively) and mock-infected cells (pSV2neo) (lane 6) was loaded in channels 1 to 6. Cos-1 cells (1×10^6) were transfected with 10 μg of plasmid DNA from clones X, ΔS, and pSV2neo and the cells harvested after 48 hours. RNA was prepared by the hot phenol approach (*21*) and 10 μg of RNA from samples X, ΔS, and pSV2neo were loaded in channels 7, 8, and 9, respectively. All samples were analyzed by Northern blotting (*17*) and hybridized to a ^{32}P-labeled probe prepared by nick translation of an Sst I–Sst I viral fragment of λBH10. Filters were washed extensively in 0.5× SSC containing 0.5% SDS at 65°C, before exposure. The positions of ribosomal 28*S* and 18*S* RNA markers are shown. Bands corresponding to 9.5 kb (genomic), 4.5 kb (envelope), and 2.0 kb viral transcripts are shown.

Table 2. Trans-activation potential of *sor* mutants of HIV. The ability of *sor* mutant genomes (ΔS, 3.3, 6.9, and 153) and full-length proviral clones (X and pHXB2gpt) to trans-activate genes linked to HIV-1 LTR elements were compared. pSV2-CAT (*12*) and pC15CAT (*13*) contain the SV40 promoter-enhancer and the HIV-1 LTR, respectively, linked to the CAT gene. pSV2-CAT served as a positive control, while pSVO-CAT (which lacks a functional promoter) was used as a negative control (*12*). The assays were performed as follows: 10 μg of plasmid DNA from pC15CAT was transfected alone or in combination with 10 μg of plasmid DNA from each of the test plasmids, into 1×10^7 H9 cells or 1×10^6 Cos-1 cells, by means of the DEAE-Dextran and calcium phosphate procedures, respectively (*14, 15*). After 48 hours the cells were harvested and washed, and the cellular lysates prepared. CAT activity was determined on 20-μl aliquots incubated with [^{14}C]chloramphenicol and Acetyl CoA for 3 hours at 37°C as described previously (*13*). Chloramphenicol and acetylated metabolites were separated by ascending thin-layer chromatography (*13*). CAT activity was determined as the counts per minute (cpm) of acetylated metabolites of chloramphenicol, expressed as a percentage of total cpm. The results shown are the means and standard deviations of three independent co-transfections for each plasmid, where each CAT assay was repeated at least twice. Using the value obtained with transfection of pC15-CAT alone as a baseline in each experiment, the results were normalized according to the following equation: f = cpm (test − pC15-CAT)/cpm (pHXB2gpt − pC15-CAT), where cpm pHXB2gpt = 1 and cpm pC15-CAT = 0.

Plasmids	Trans-activation in H9 cells		Trans-activation in Cos-1 cells	
	Mean conversion (%)	f value	Mean conversion (%)	f value
pHXB2gpt	40.5 ± 3.9	1.0	56.1 ± 19.9	1.0
ΔS	32.0 ± 6.9	0.89	67.5 ± 28.9	1.22
3.3	43.0 ± 8.9	1.05	62.0 ± 17.5	1.24
6.9	54.3 ± 23.9	1.2	37.5 ± 15.5	0.66
153	29.8 ± 2.5	0.86	47.0 ± 25.7	0.8
pC15CAT	0.5 ± 0.1	0.0	2.0 ± 1.0	0.0
pSV2CAT	17.4 ± 2.7	0.55	44.4 ± 6.2	0.83

corresponds to *gag*-derived p39. Alternatively, this band may represent the closely migrating transmembrane product of *env* (gp41). The presence of comparable amounts of virus-specific proteins in samples 3.3, 6.9, and X (lanes 2, 3, and 4, respectively) was confirmed by end-point titration. Immunoprecipitation of Molt 3 cell lysates with rabbit antiserum to *sor* (*10*) showed a virus-specific band corresponding to p23-*sor* in samples carrying the wild-type strain X (lane 5). No equivalent band was seen in uninfected cells or cells infected with mutant 6.9 or 3.3 virus (lanes 8, 6, and 7, respectively). Failure to detect a truncated *sor* gene product in the case of 6.9 and 3.3 suggests that the antiserum recognized either an epitope downstream of residue 55 (the position of the stop codon in 6.9) or a conformational epitope within the first 55 residues (altered in the truncation), or that the truncated proteins were extremely labile. In parallel experiments with a rabbit antiserum to *sor*, we were unable to detect normal (p23) or truncated *sor* products from Cos-1 transfected cells, H9 transfected cells, or even H9/HTLV-III$_B$ cultures (which produce high levels of infectious virus). *Sor* protein was, in our hands, detected only in Molt 3 infected cell lines. These findings suggest that *sor* may only be present, in a stable form, at low levels of HIV-1 infected cells and that *sor* expression might, in part, be influenced by cellular factors.

Cellular lysates of Cos-1 cells transfected with *sor* mutant genomes were also analyzed for HIV protein expression (Fig. 5, C and D). Immunoprecipitation with sera from AIDS patients showed a distinct band at 39 to 41 kD in lysates of cells transfected with ΔS, 3.3, 6.9, 153 (lanes 12, 13, 14, 15), X (lanes 9 and 16), and pHXB2gpt (lane 11). This band was absent from control lysates of cells transfected with pSP65gpt (lanes 10 and 17), a clone devoid of HIV-1 sequences. A 55-kD band (sample X, lane 9), corresponding to an alternative *gag* precursor, was frequently seen in Cos-1 transfected lysates (although it is not evident in Fig. 5D, lanes 11 to 17). *Gag*-derived p24 was evident in all cultures although the amount detected varied slightly between and within experiments (see differences in lanes 9, 11, and 16; samples transfected with intact proviral clones). In repeated experiments we were unable to show reproducible differences in the amounts of viral proteins (above those attributable to technical fluctuations) between samples that had been transfected with *sor* mutant or intact proviral clones. These data indicate that normal levels of HIV-1 *gag*- and *env*-derived proteins are produced in Molt 3 cells and Cos-1 cultures carrying the *sor* mutant viruses and are in agreement with the previous report (*6*).

We next directly assessed the ability of *sor* mutant genomes to trans-activate viral LTR sequences. The plasmid pC15CAT, which contains the bacterial chloramphenicol acetyltransferase (CAT) gene linked to the HIV-1 LTR's, was used as an indicator. Transfections were performed in lymphoid and nonhemopoietic cells (H9 and Cos-1 cell lines, respectively). As shown in Table 2, trans-activation activity occurred with each of the *sor* mutant genomes, at levels approximating that of wild type. In H9 cells transfected with *sor* mutants, the mean conversions were 54%, 43%, 32%, and 30% (6.9, 3.3, ΔS, and 153, respectively), compared with 41% for wild type (pHXB2gpt). This represents a range of 86 to 120% of the values obtained for pHXB2gpt. Similar results were obtained from Cos-1 transfections where the values for *sor* mutants were 66 to 124% of those obtained for pHXB2gpt. Trans-activation levels were slightly higher and more variable in Cos-1 transfected cells than H9 transfected cells. Thus the trans-acting potential of the *sor* point mutants (3.3, 6.9, 153) and deletion mutant (ΔS) is comparable to wild type and suggest that *sor* function is independent of trans-activation.

Our data thus show that removal or truncation of *sor* results in virus progeny that have a much reduced (>100-fold) capacity to infect CD4$^+$ cells. Furthermore, *sor* mutant viruses were transmitted less well under coculture conditions (in which cell to cell transmission is likely to be important). It is interesting that the effects of truncating *sor* (in the case of mutants 3.3, 6.9, 153) were similar to its complete removal (in the case of mutant ΔS), suggesting that the carboxyl terminal portion of *sor* (downstream from residue 100) may include a functional domain. However, the possibility that *sor* is nonfunctional because it is deprived of critical elements in the carboxyl terminal of the protein necessary for the correct folding or processing is not excluded. Since the level of viral RNA, proteins, and viral particles produced by *sor*-defective genomes could not be distinguished from that of wild type, we suspect that *sor* exerts its effects at a post-translational level. This novel regulatory mechanism, mediated by *sor*, could involve late events in virus maturation. This is, to our knowledge, the first report in which a function for the *sor* gene of HIV-1 has been assigned. These findings substantiate the complex nature of the HIV-1 genome, underscoring the sophisticated transcriptional, post-transcriptional, and post-translational controls which operate to regulate virus expression in infected cells. Although we do not yet understand how *sor* enhances virus propagation, several mechanisms are possible. For example, *sor* may be a structural component of the virion particle that acts as a "second envelope" required for efficient transmission. However, it is difficult to de-

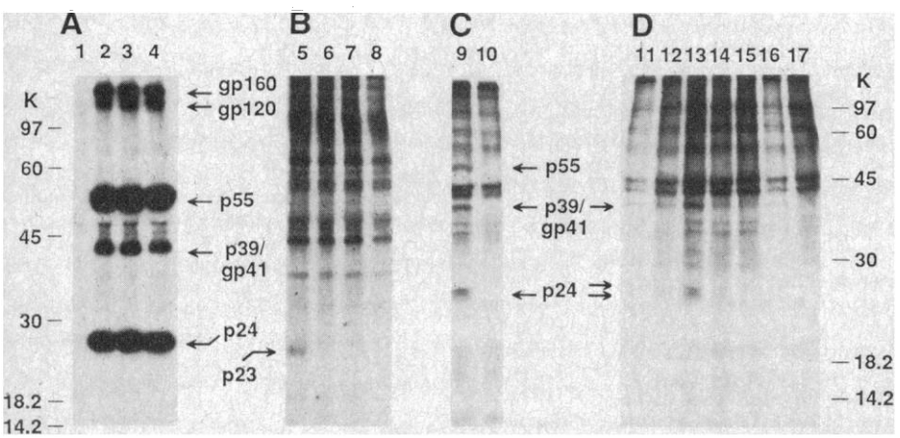

Fig. 5. Radioimmunoprecipitation analysis of HIV-1–specific proteins produced by *sor* mutant genomes. Cos-1 cells (1×10^6) were transfected with 10 μg of plasmid DNA by using the calcium phosphate technique (*14*). At 48 hours after transfection, [^{35}S]methionine (200 μCi/ml) was added to the cultures and the cells were incubated for a further 6 hours at 37°C. Molt 3 cells (5×10^6) originating from coculture experiments (see Fig. 3) containing >90% HIV-1–infected cells were also labeled with [^{35}S]methionine (200 μCi/ml) for 6 hours. Cellular extracts were prepared and immunoprecipitated with serum obtained from an AIDS patient (lanes 1 through 4 and 9 through 17) or rabbit antiserum to *sor* (*10*) (lanes 5 through 8), and the proteins were separated by SDS-PAGE (10%) as described elsewhere (*22*). The results from four experiments are shown. (**A** and **B**) Molt 3 cell lysates from 3.3- (lanes 2 and 7), 6.9- (lanes 3 and 6), and X- (lanes 4 and 5) infected cells. Virus-negative, mock-infected (pSV2neo) Molt 3 cell lysates are shown in lanes 1 and 8. (**C** and **D**) Results with Cos-1–derived lysates. Cos-1 cells transfected with X (lanes 9 and 16), pHXB2gpt (lane 11), ΔS (lane 12), 3.3 (lane 13), 6.9 (lane 14), and 153 (lane 15) are shown, as are samples transfected with control plasmid pSP65gpt (lanes 10 and 17). The position of HIV-1–specific gp160, gp120, p55, p39/41, and p24 is indicated as is the p23 (*sor*) seen only in lane 5.

tect *sor* in as large amounts as gp120 and gp41 (in either infected cells or virions). Alternatively, *sor* may participate in the early events in viral replication (from penetration to synthesis of proviral DNA) or in potentiating the cellular environment in which replication occurs. Further studies will be necessary to evaluate these possibilities.

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20. The presence of each mutation within the new constructs was verified by Southern blotting analysis using the oligonucleotides as probes and in conditions in which the mutated and nonmutated genomes could be distinguished. Plasmids ΔS, 3.3, 6.9, 153, and X, and the parental clone pHXB2gpt were transfected into OKT4$^+$ H9 cells by the protoplast fusion approach (*7*). The transfected cultures were maintained in RPMI 1640 medium containing 20% fetal calf serum and antibiotics for 4 to 6 weeks and analyzed weekly for the presence of HIV-1 *gag* (p17, p24), envelope (p41), and virus particles. HIV-1 *gag* and *env* expression was visualized by using the monoclonal antibodies BT2, BT3, and M25 [F.d: M. Veronese *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5199 (1985)], fluorescein conjugated sheep antibody to mouse immunoglobulin, and standard immunofluorescence approaches. The RT assays were performed on tenfold concentrated spent culture medium by routine procedures (*11*) with Mg^{2+} being used as a cofactor. Cultures were judged to be HIV-1 infected if they satisfied any of the following criteria: (i) HIV-1 *gag*/*env* expression could be reproducibly detected in cultures at frequencies exceeding 0.1%; (ii) RT assays performed on at least two sequential samples had values for ^{3}HdTMP incorporated, three or more times background levels; (iii) virus particles were detected by electron microscopy.
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A Parathyroid Hormone–Related Protein Implicated in Malignant Hypercalcemia: Cloning and Expression

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Humoral hypercalcemia of malignancy is a common complication of lung and certain other cancers. The hypercalcemia results from the actions of tumor factors on bone and kidney. We report here the isolation of full-length complementary DNA clones of a putative hypercalcemia factor, and the expression from the cloned DNA of the active protein in mammalian cells. The clones encode a prepro peptide of 36 amino acids and a mature protein of 141 amino acids that has significant homology with parathyroid hormone in the amino-terminal region. This previously unrecognized hormone may be important in normal as well as abnormal calcium metabolism.

HYPERCALCEMIA IS FREQUENTLY associated with malignant disease. Humoral hypercalcemia of malignancy (HHM) occurs in cancer patients without bony metastases often in association with squamous cell carcinoma of the lung, where it is a major contributor to morbidity and complicates clinical management (*1–3*). The hypercalcemia is caused by tumor products acting on bone to promote resorption and on the kidney to restrict calcium excretion (*2–4*). The biochemical similarities between primary hyperparathyroidism and the HHM syndrome (*2, 5*) pointed to the likelihood that these tumors produce a substance that has actions very similar to parathyroid hormone (PTH). This tumor factor is distinct from PTH, however, since PTH radioimmunoassays usually fail to detect increased levels of the hormone in plasma from HHM subjects (*2, 3, 6*) and since PTH messenger RNA is not found in the tumors of such patients (*7*). Using a bioassay based on the stimulation of adenosine 3′,5′-monophosphate (cAMP) levels in the PTH-responsive rat osteogenic sarcoma cell line UMR106-01, we have recently purified a protein of M_r 18,000 from the conditioned medium of a human lung cancer cell line (BEN) derived originally from a patient with HHM syndrome (*8*). Similar or identical biological activities have also been identified in extracts of tumors from HHM patients (*9, 10*), from animal tumor models of the syndrome (*10, 11*), and from conditioned media from cultures of two such tumors (*8, 12*). The amino acid sequence of the first 16 residues of the BEN cell–derived protein has been determined, and 8 of the 16 residues are identical with human PTH (*8*). We describe here the isolation of complementary DNA (cDNA) clones, the complete primary structure, and the active expression in mammalian cells of the PTH-related protein from BEN cells.

Clones of the PTH-related protein were isolated from a cDNA library of BEN cell RNA by screening with oligonucleotide probes based on NH_2-terminal sequence data obtained from the purified protein (*13*). Two 72-base oligonucleotides were synthesized corresponding to a 24–amino acid NH_2-terminal sequence (*8*); one used codons based on mammalian frequency tables (*14*) and the second used codons from PTH for the positions of amino acid match. A total of 250,000 primary, oligo(dT)-primed, cDNA clones in the vector λgt10 were screened with a mixture of the two

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