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Notes:

# Human immunodeficiency virus tat-activated expression of poliovirus protein 2A inhibits mRNA translation

(trans-activation/protein synthesis/protease)

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**ABSTRACT** To study the effect of poliovirus protein 2A on cellular RNA translation, the tat control system of human immunodeficiency virus (HIV) was used. Protein 2A was expressed from a plasmid construct (pHIV/2A) incorporating the HIV long terminal repeat. Protein synthesis was measured by using chloramphenicol acetyltransferase as a reporter gene driven by the Rous sarcoma virus long terminal repeat. When HIV/2A was cotransfected with the reporter, addition of a tat-producing plasmid caused at least a 50-fold drop in chloramphenicol acetyltransferase synthesis. A HeLa cell line carrying HIV/2A was established. In it, tat expression caused more than a 10-fold drop in chloramphenicol acetyltransferase synthesis from the reporter plasmid. Furthermore, 2A induction by tat caused cleavage of the cellular translation factor P220, a part of eukaryotic translation initiation factor 4F. Thus protein 2A can, by itself, carry out the inhibition of cellular protein synthesis characteristic of a poliovirus infection. Also, the HIV tat activation provides a very effective method to control gene expression in mammalian cells.

Poliovirus causes a dramatic shutoff of host cell mRNA translation within several hours after infection (1). The mechanism of shutoff is thought to be inactivation of eukaryotic translation initiation factor 4F (eIF-4F) (2). Poliovirus induces the cleavage of the P220 component of eIF-4F (3), a factor that assists the binding of capped mRNAs to the 40S ribosome as part of the translation initiation process (4). The virus's strategy thus appears to be to prevent the cap-dependent translation initiation used by cellular mRNAs, while allowing the cap-independent translation of polioviral RNA.

Poliovirus protein 2A is a protease whose mature form is generated by the cleavage of the precursor polyprotein of poliovirus at its N terminus by 2A itself (5) and at the C terminus by another protease, 3C (6). In addition to its cleavage function, 2A also appears to be involved in the shutoff of cellular protein synthesis, because an insertion in the protein 2A yields a small-plaque mutant virus defective in the shutoff (7). Infection with such mutant virus also fails to induce the cleavage of P220 (7), which occurs during wild-type virus infection as well as in HeLa cell extracts in the presence of *in vitro*-translated 2A protein (8). We have used the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) as a controlling element for expression of protein 2A in HeLa cells and found that 2A by itself dramatically inhibits cellular mRNA translation. We also detected cleavage of P220 following 2A expression. Additionally, we have shown that the LTR of HIV-1 and its trans-activator (tat protein) can act as an effective, controlled expression system.

## MATERIALS AND METHODS

**Plasmids.** The structures of the plasmids are shown in Fig. 1. To generate pHIV/2A, a *Bst*EII fragment (nucleotides 3235–3930) from a poliovirus cDNA (9) was filled in with DNA polymerase (Klenow fragment), attached to a 12-mer *Eco*RI linker, and then inserted into the *Eco*RI site of the chloramphenicol acetyltransferase (CAT) gene driven by HIV-1 LTR in a plasmid, called 933A (a gift of Sunyoung Kim, Whitehead Institute for Biomedical Research). This fragment is in-frame with the CAT gene at its N terminus, but out-of-frame at its C terminus, which creates a stop codon following 9 random amino acids. Thus, the coding sequence of the fusion gene contains 73 N-terminal amino acids of CAT, the polioviral sequence including 50 C-terminal amino acids of region P1, the entire 2A, and 33 N-terminal amino acids of protein 2B, and the 9 random amino acids. In the transcription unit of pHIV/2A, between the stop codon and the simian virus 40 (SV40) polyadenylation site, are a 3' portion of the CAT gene, 900 base pairs of truncated envelope sequence of HIV-1, and the intervening sequence of SV40 (not indicated in Fig. 1). pHIV/2A-1 was constructed by replacing the *Bst*EII fragment in pHIV/2A with the *Bst*EII fragment from the 2A-1 mutant cDNA (7, 10). To generate the deletion in 2A of pHIV/2APX, we digested HIV/2A plasmid with *Xba* I (nucleotide 3581 in the poliovirus sequence) and filled in the *Xba* I site. This linear DNA was then partially digested with *Pst* I, and the fragment that was digested only at the *Pst* I site in the poliovirus sequence (nucleotide 3420) was selected by using a 1% low-melting-point agarose gel. The two ends of this fragment were ligated with a polylinker fragment with a *Pst* I end on one side and a blunt end on the other, which was isolated from the Bluescript plasmid (Stratagene) by digesting with *Pst* I and *Eco*RV.

***In Vitro* Transcription and Translation.** To synthesize RNAs *in vitro*, the *Hind*III–*Bgl* II fragments (Fig. 1) containing the coding sequences of the fusion proteins were excised from HIV/2A, HIV/2A-1, and HIV/2APX and ligated to the *Hind*III and *Bam*HI sites of SP64 (Promega). A *Hind*III–*Bgl*II fragment containing the CAT reading frame was also cloned into SP64 to synthesize a control template. The coding strands were synthesized with SP6 polymerase (Promega). Approximately 1  $\mu$ g of RNA was used for the *in vitro* translation in 50  $\mu$ l of the nuclease-treated rabbit reticulocyte lysate (Promega) containing [<sup>35</sup>S]methionine according to the vendor. Five microliters of each reaction mix was diluted in 1 ml of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/1 mM EDTA) and centrifuged at 40,000 rpm in an SW50.1 rotor (Beckman) for 30 min. The supernatants were immunoprecipitated with an anti-2A serum and *Staphy-*

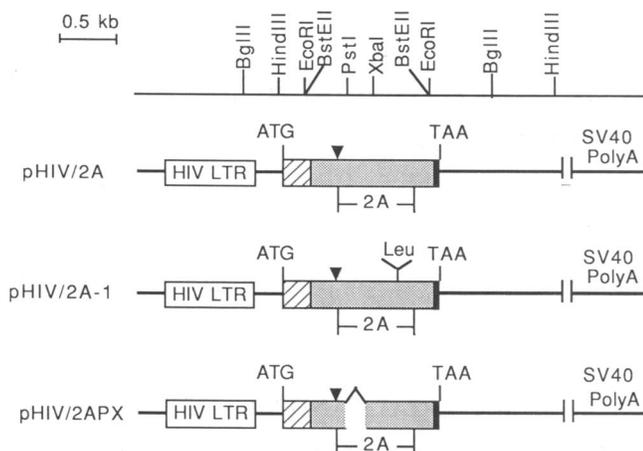


FIG. 1. Construction of the HIV/2A gene and derivatives. A restriction map of HIV/2A is shown at the top. The LTR of HIV-1 is boxed. The coding sequence of HIV/2A or its derivatives is shown by the second box, in which the CAT sequence, the polioviral sequence, and random amino acids are represented by the hatched, stippled, and solid black portions, respectively. The region in the polioviral sequence encoding 2A is bracketed underneath. The junction of P1 and 2A is marked by an inverted triangle. The remainder of the sequences are shown with a thin line, and the SV40 polyadenylation site is indicated. kb, Kilobase.

*lococcus aureus* protein A-positive cells (Boehringer Mannheim). Portions of the supernatants and pellets were analyzed by electrophoresis through a SDS/20% polyacrylamide gel.

**Transfection of HeLa Cells and Establishing Cell Lines.** HeLa cells were plated in Dubecco's modified Eagle's medium containing 10% fetal calf serum at about  $5 \times 10^5$  cells per 60-mm Petri dish 12–24 hr prior to transfection. The calcium phosphate/DNA precipitates were prepared as described (11) and applied to cells without the medium and incubated at 25°C for 15 min before addition of fresh medium. Glycerol shock was performed 12 hr after the addition of precipitates. Cells were harvested 48 hr after the addition of precipitates, and CAT activities were assayed as described (12). The HeLa 2A-38 cell line was established by cotransfecting pHIV/2A with pSV2neo (13) by using the calcium phosphate/DNA precipitate method. Transfected cells were selected 48 hr later with G418 at 1 mg/ml in Dulbecco's modified Eagle's medium for 2 weeks. About 50 individual colonies were propagated for analyzing their genomic structures and the inhibitory function of 2A.

**Protein Immunoblot.** An immunoblot experiment was performed with an antiserum against P220, essentially as described by Bernstein *et al.* (7) except that an alkaline phosphatase-conjugated anti-rabbit IgG (Promega) was used as the second antibody as described by the vendor.

## RESULTS

### Constructs Expressing 2A Under the Control of HIV LTR.

To express the poliovirus protein 2A in cells, we constructed a plasmid, pHIV/2A, by fusing a 695-base-pair *BstEII* fragment from a poliovirus cDNA (Mahoney strain) in frame with the coding sequence of a CAT gene, whose expression is under the control of the HIV-1 LTR (Fig. 1). We also constructed similar fusion genes, pHIV/2A-1 and pHIV/2APX, with mutations in the 2A sequence (Fig. 1). pHIV/2A-1 contains a single amino acid insertion in 2A, which results in a mutant poliovirus, 2A-1, that is defective in shutting off host cell protein synthesis upon infection (7, 10). pHIV/2APX has a 53-amino acid deletion in 2A.

**Proper Processing of the 2A Fusion Proteins.** Because 2A, which has a proteolytic activity, is known to cleave the

junction between P1 and itself (5), we expected that the predicted 41-kDa fusion protein from the constructs would be processed by 2A, resulting in a mature protein of 24.5 kDa containing only 2A and a small part of 2B. We demonstrated such processing by examining the *in vitro*-translated products from the coding sequences of the fusion proteins in various constructs. The RNA containing the coding sequence of pHIV/2A directed synthesis of a 24.5-kDa protein (Fig. 2a, lane 3) that was immunoprecipitated by anti-2A serum (Fig. 2b, lane 3). The coding sequence should direct synthesis of a 41-kDa protein, but processing is apparently so complete that none is left; the 17-kDa P1 cleaved polypeptide was not detected and may have been degraded. The partially defective mutant 2A-1 gave rise to 24.5-kDa and 41-kDa products, both of which reacted with anti-2A serum (Fig. 2, lanes 4), implying that mutant 2A-1 is defective in cleavage of 2A from its precursor and confirming that a 41-kDa product is made from the construct. In pHIV/2APX, the 53-amino acid deletion caused synthesis of a major 33-kDa immunoprecipitable product (Fig. 2, lanes 5). The minor 27-kDa product is presumed to result from alternative initiation from an in-frame AUG or could be an aberrant nonspecific cleavage product. Specific cleavage at the P1/2A junction would have generated a much smaller product. Thus, the constructs produce the predicted products, implying that 2A is proteolytically active and that the mutants partially or completely block specific cleavage.

**Effects of 2A on Cellular mRNA Translation.** We then examined the ability of 2A to inhibit cellular mRNA translation by cotransfection experiments. The plasmids used were a reporter construct, pRSVCAT (14), in which the CAT gene is controlled by the LTR of Rous sarcoma virus; pHIV/2A, expressing poliovirus protein 2A; and pSVETA, in which the HIV-1 *tat* gene is transcribed from an SV40 promoter (15), providing trans-activator function (Fig. 3c). We expected that pHIV/2A would need *tat* synthesis from pSVETA for expression of the 2A protein because the HIV LTR is strongly controlled by *tat* (15). We also expected that the expression of 2A would inhibit the translation of pRSVCAT mRNA and give a low CAT activity (Fig. 3c). These expectations were fulfilled: the expression of *tat* greatly decreased synthesis of CAT when cells were cotransfected with pRSVCAT and pHIV/2A (Fig. 3a, compare lanes 3 and 4). Without pHIV/2A, no inhibition was evident (Fig. 3a, lanes 1 and 2). The single amino acid insertion in 2A greatly impaired the ability of 2A to inhibit mRNA translation (Fig. 3a, lanes 5 and 6), whereas a deletion in 2A completely abolished its inhibitory function (Fig. 3a, lanes 7 and 8). In addition, we performed similar cotransfection experiments with a pRSVLacZ plasmid (the  $\beta$ -galactosidase gene driven by the LTR of Rous sarcoma virus) (16) or a pSV2CAT

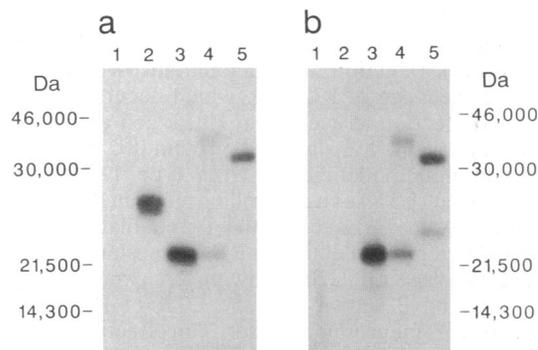


FIG. 2. *In vitro* translation of the 2A and 2A mutant fusion proteins. (a) Total translation products. (b) Immunoprecipitation. RNAs used for translation contain the coding sequences of mock (lane 1), CAT (lane 2), 2A (lane 3), 2A-1 (lane 4), and 2APX (lane 5).

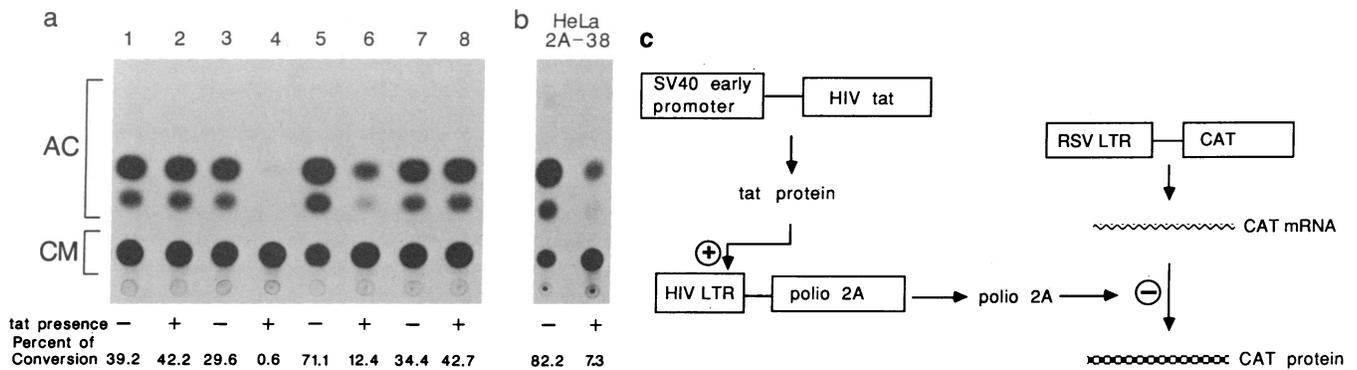


FIG. 3. The inhibitory effect of 2A on mRNA translation. (a) HeLa cells were cotransfected with equal molar amounts of pRSVCAT and either pSVETA or carrier DNA as indicated plus carrier DNA (lanes 1 and 2), pHIV/2A (lanes 3 and 4), pHIV/2A-1 (lanes 5 and 6), and pHIV/2APX (lanes 7 and 8). CAT activities were measured and expressed as the percentage of conversion from chloramphenicol (CM) to acetylated chloramphenicol (AC). (b) In the HeLa 2A-38 cell line, 10  $\mu$ g of pRSVCAT was cotransfected with or without 10  $\mu$ g of pSVETA, and CAT activities were determined. The above experiments have been repeated at least three times, and similar results were obtained. (c) Rationale of the experimental design.

plasmid (the CAT gene driven by SV40 early promoter) (12) as the reporter genes and obtained equivalent results (data not shown), suggesting that the inhibition of translation by 2A is not specific to a particular protein, promoter, or mRNA capping sequence. Thus, we conclude that 2A, even when it is expressed out of the context of poliovirus, inhibits cellular mRNA translation. It probably will kill cells, although that is not demonstrated here.

**Establishment of the Cell Lines Carrying HIV/2A Gene.** The low level of expression from the LTR of HIV-1 without *tat* expression allowed us to establish HeLa cell lines permanently carrying pHIV/2A. The plasmid pHIV/2A was cotransfected into HeLa cells with the plasmid pSV2neo, which expressed the neomycin-resistance gene (13). Several dozen individual neomycin-resistant colonies were obtained. The expression of the integrated HIV/2A genes could then be activated by transfecting in the *tat* gene expressed by pSVETA. The inhibitory function of 2A in these cell lines was assayed by cotransfecting pRSVCAT and pSVETA and measuring the CAT activity of the cotransfected extracts. These cell lines were found to contain variable copies of HIV/2A genes and respond to *tat* activation to variable extents. One of the cell lines, 2A-38, carried 10–20 copies of the HIV/2A gene and translated RSVCAT mRNA more than

10 times less efficiently when pRSVCAT was cotransfected with pSVETA compared to transfection without pSVETA (Fig. 3b).

**Cleavage of P220 by 2A Expression *in Vivo*.** During poliovirus infection, not only is host cell protein synthesis shut off, but also the polypeptide P220, a component of eIF-4F, is cleaved (3). Poliovirus protein 2A also appears to be involved in this cleavage event, both because mutant 2A-1 fails to trigger the cleavage of P220 (7) and because *in vitro*-translated 2A indirectly induces the cleavage of P220 in a HeLa cell extract (8). We therefore investigated if 2A, when expressed alone *in vivo*, could cause the cleavage of P220. We activated the expression of 2A in the cell line 2A-38 by transiently transfecting it with the *tat* gene and assayed the cleavage products of P220 in the transfected extract by an immunoblot using an anti-P220 serum. The cleavage products were apparent in the induced 2A-38 cells (Fig. 4, lane 3) but not in either the unactivated 2A-38 cells (lane 4) or normal HeLa cells (Fig. 4, lanes 1 and 2). The cleaved products comigrated with those in poliovirus-infected HeLa cells (Fig. 4, lane 6). Due to the inefficiency of the calcium phosphate/DNA precipitate transfection method, only a portion of the 2A-38 cells used for transfection would be expected to receive the *tat* gene, express 2A, and induce the cleavage of P220. Thus, in Fig. 4, lane 3, the cleavage products were probably contributed from this portion of the cells, whereas the intact P220 would be from the remaining portion that did not receive the *tat* gene. Fig. 4, lane 4 also emphasizes that in 2A-38 cells the baseline expression of 2A protein is so low that no cleavage of P220 is detectable.

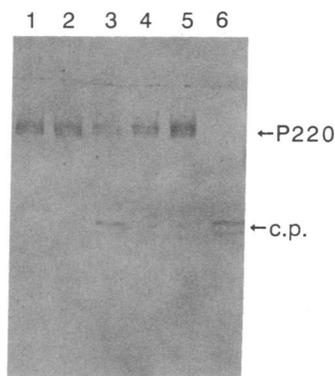


FIG. 4. The cleavage of P220 in 2A-producing cells. Immunoblot experiment was performed with an antiserum against P220. Extracts were prepared 48 hr posttransfection from HeLa cells transfected with or without pSVETA (lanes 1 and 2) and 2A-38 cells transfected with or without pSVETA (lanes 3 and 4). Extracts were also prepared from HeLa cells infected with wild-type (lane 6) or 2A-1 mutant virus (lane 5) at multiplicity of infection of 20 for 3.5 hr. One-fifth of the extracts from each 60-mm dish was analyzed by electrophoresis through a SDS/6.5% polyacrylamide gel. P220 and its cleavage products (c.p.) are indicated.

## DISCUSSION

It was previously evident that a mutant in protein 2A interferes with the virus-induced shutoff of host cell protein synthesis (7). It was also evident that 2A is a protease that cleaves the P1–2A bond in the poliovirus polyprotein (5). Because protein 2A is cleaved from precursors, it was not clear that 2A itself was responsible for both effects. The present results show directly that expression of 2A (with a short tail of 2B attached) can cause inhibition of cellular mRNA translation. 2A also acts as a protease, since 2A mutants are defective in cleavage of 2A from its precursor. 2A is able to induce cleavage of P220, as was also evident from *in vitro* results (8), but the mechanism of cleavage, thought to be an indirect effect of 2A (8), remains obscure.

Unlike most other retroviruses, the LTR of HIV can be transactivated several hundred-fold by the *tat* gene from a very low basal level (15, 17). The level of expression in HeLa

cells after activation is much higher than synthesis from Rous sarcoma virus LTR or SV40 early promoter. We have shown, by our success in establishing cell lines carrying the HIV/2A gene, that this feature of the HIV LTR can be utilized as a good activatable system to study the functions of proteins toxic to cells. On the other hand, this feature can also be utilized to design strategies that might counter HIV infection. For example, by introducing the HIV/2A gene into cells before or after HIV infection, we could specifically kill the infected cells and prevent further infection of normal cells.

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1. Baltimore, D. (1969) in *The Biochemistry of Picornaviruses*, ed. Levy, H. (Dekker, New York), pp. 101-176.
2. Sonenberg, N. (1987) *Adv. Virus Res.* **33**, 175-204.
3. Etchison, D., Milburn, S., Edery, I., Sonenberg, N. & Hershey, J. W. (1982) *J. Biol. Chem.* **257**, 14806-14810.
4. Shatkin, A. (1985) *Cell* **40**, 223-224.
5. Toyoda, H., Nicklin, M. J. H., Murry, M. G. & Wimmer, E. (1986) *Cell* **45**, 761-770.
6. Hanecak, R., Semler, B. L., Anderson, C. W. & Wimmer, E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3973-3977.
7. Bernstein, H. D., Sonenberg, N. & Baltimore, D. (1985) *Mol. Cell. Biol.* **5**, 2913-2923.
8. Krausslich, H. G., Nicklin, M. J. H., Toyoda, H., Etchison, D. & Wimmer, E. (1987) *J. Virol.* **61**, 2711-2718.
9. Racaniello, V. R. & Baltimore, D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4887-4891.
10. Bernstein, H. D., Sarnow, P. & Baltimore, D. (1986) *J. Virol.* **60**, 1040-1049.
11. Sun, X.-H., Lis, J. T. & Wu, R. (1988) *Genes Devel.* **2**, 743-753.
12. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044-1051.
13. Southern, P. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1**, 327-341.
14. Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. & Howard, B. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6777-6781.
15. Muesing, M. A., Smith, D. H. & Capon, D. J. (1987) *Cell* **48**, 691-701.
16. Hall, C. V., Jacob, P. E., Ringold, G. M. & Lee, F. (1983) *J. Mol. Appl. Genet.* **2**, 101-109.
17. Sodroski, J., Rosen, C., Wong-Staal, F., Salahuddin, S. Z., Popovic, M., Arya, S., Gallo, R. C. & Haseltine, W. A. (1985) *Science* **227**, 171-173.