Expression of the *art* Gene Protein of Human T-Lymphotropic Virus Type III (HTLV-III/LAV) in Bacteria

WEI CHUN GOH,¹ JOSEPH G. SODROSKI,¹ CRAIG A. ROSEN,¹ AND WILLIAM A. HASELTINE^{2*}

Dana-Farber Cancer Institute¹ and Harvard School of Public Health,² Boston, Massachusetts 02115

Received 16 July 1986/Accepted 7 October 1986

Human T-lymphotropic virus type III (HTLV-III/LAV or HIV) contains a gene designated *art* (anti-repressor transactivator). Here, we report the expression of the *art* gene product in bacteria and show that the 20-kilodalton (kDa) bacterially expressed *art* protein is recognized by serum of a patient. The bacterially synthesized *art* protein competed in an immunological reaction with a 20-kDa protein produced in HTLV-III/LAV-infected lymphocytes. Antiserum to a synthetic oligopeptide corresponding to a sequence in the second exon of the *art* gene also precipitated the 20-kDa protein in HTLV-III/LAV-infected cells. These results demonstrate that the 20-kDa *art* gene product is expressed in cell lines that produce HTLV-III/LAV virions.

Human T-lymphotropic virus type III (HTLV-III/LAV or HIV), a retrovirus, is the etiological agent of the acquired immune deficiency syndrome (AIDS) and other related disorders (3, 6, 8, 10, 12, 13, 16-18). In addition to the *gag*, *pol*, and *env* genes encoded by other retroviruses, the genome of HTLV-III has been shown to encode at least three additional proteins: the 23-kilodalton (kDa) *sor* (9, 11, 20), the 14-kDa *tat*III (7), and the 27-kDa 3' *orf* gene products (1). Of the additional genes, the *tat*III gene, but not the *sor* and 3' *orf* gene functions, is required for viral growth and cytopathicity in T4+ cell lines (4, 5, 20). Recent evidence indicates that HTLV-III encodes an additional gene, termed *art*, that is also required for virus replication (19). Transfection of cells with mutant proviruses defective in the *art* gene results in the production of viral RNA and a functional *tat*III gene product but not the structural capsid or envelope gene product. The postulated boundaries of the *art* gene are shown in Fig. 1. This region consists of two coding exons which partly overlap the first and second exons of *tat*III in an alternative reading frame. The initiation codon of the putative *art* gene protein is located at nucleotide 5550 (14), 3' to the initiation codon of the *tat*III gene product. Plasmids with an HTLV-III viral long terminal repeat placed 5' to the *art* gene initiation codon will complement in *trans* proviruses with mutations in the *art* gene (19). Disruption of this alternative reading frame by a frameshift mutation effectively abolishes *art* gene function (19). For these reasons, it was proposed that the *art* gene encodes a polypeptide.

The ability of the *art* gene to encode a protein was examined by determining whether a polypeptide synthesized in bacteria corresponding to the *art* region is capable of competing for recognition by naturally occurring patient antiserum of a virally encoded protein in an immunoprecipitation reaction. To express the *art* gene product in bacteria, the *art* open reading frame present on a cDNA species (2) that contains the correctly spliced exons of the *art* gene was inserted into an inducible bacterial overexpression vector, pAS1 (15), as described in the legend to Fig. 2. Expression in

this plasmid is driven by the bacteriophage lambda promoter (*p_L*) that is normally repressed by the lambda *cI* repressor gene. The phage lambda *cII* gene provides an ATG codon appropriately placed for efficient translational initiation. Genes fused in frame with this methionine codon were monitored for protein expression in a bacterial strain (N88cI^{ts857}) that harbors a prophage with a temperature-sensitive *cI* gene. Three particular clones, pAS1-ART1, pAS1-ART2, and pAS1-ART3 (Fig. 2), were examined for protein production in *Escherichia coli*. Plasmid pAS1-ART1 removes the ATG codon from the amino terminus of *art* and replaces it with three amino acids, Met-Asp-Leu. Plasmid pAS1-ART2 replaces in frame the first three amino acids, Met-Ala-Gly, from the amino terminus of the *art* open reading frame with three new amino acids, Met-Asp-Leu. However, plasmid pAS1-ART3 contains an out-of-frame fusion with the *art* coding region.

Protein production by the three clones was determined in *E. coli* N99cI^{ts857} by pulse-labeling for 1 min with [³H]arginine either before or after temperature induction. At 42°C, a novel protein of approximately 20 kDa was induced in *E. coli* strains that contained either the pAS1-ART1 or pAS1-ART2 plasmid but not in the out-of-frame pAS1-ART3 plasmid, indicating that the 20-kDa protein was synthesized from the *art* frame (Fig. 3A). No translation initiation methionine codon other than the ATG provided by the *cII* gene is present in the *art* region of the cDNA clone used.

To determine whether sera from HTLV-III-infected persons contain antibodies to the bacterially synthesized 20-kDa protein, labeled bacterial extracts were used for immunoprecipitation analysis with sera from AIDS patients. The 20-kDa protein induced at 42°C from pAS1-ART1 and pAS1-ART2 was recognized by sera from patients (Fig. 3B). Of five different sera tested, two were positive.

One of the sera that precipitated the 20-kDa protein was tested for its ability to recognize proteins produced in HTLV-III-infected cells. Protein extracts were prepared from [³H]arginine- and [³⁵S]cysteine-labeled H9/HTLV-IIIIB cells, a virus-producing T-lymphocyte cell line. The serum contained antibodies that reacted with a variety of viral proteins including gp160, gp120 envelope gene products, and p55, p24-25, and p17 *gag* gene products, as well as the 27-kDa 3' *orf* gene product (Fig. 4). In addition, a 20-kDa

* Corresponding author.

FIG. 1. Structure of the *art* gene of HTLV-III. Panel A shows the 3' half of the HTLV-III genome, with the relative positions of the *tat*III, *art*, *env*, and 3' *orf* genes depicted. The vertical broken line in the 3' *orf* gene indicates the position of a stop codon found in some cloned HTLV-III proviral genomes. Numbers refer to the nucleotide sequence of Ratner et al. (14). Panel B shows in detail the open reading frames surrounding the *tat*III and *art* genes, with the vertical lines representing the positions of stop codons. The nucleotide sequence and the predicted amino acid sequence of the *art* gene are also depicted.

FIG. 2. Bacterial overexpression vector for the *art* protein. The *art* coding region was obtained from a cDNA clone (2) by treating the cDNA clone with BAL 31 after digestion with *Sal*I. *Bgl*II linkers were then ligated to the digested ends, and the plasmid was further digested with *Xho*I. *Bgl*II-*Xho*I fragments containing the *art* coding sequence were then inserted into the pAS1 vector (15), which had been digested with *Bam*HI and *Sal*I. All cloning work was done in *E. coli* N99c1+, a lambda lysogen. The endpoints of the coding frame for the *art* sequence were determined by Maxam-Gilbert sequencing. Shown at the bottom of the figure are the sequences of two in-frame plasmids, pAS1-ART1 and pAS1-ART2, and an out-of-frame clone, pAS1-ART3.

protein not present in the immunoprecipitates of uninfected H9 cells is evident in this experiment. To determine whether the 20-kDa protein present in HTLV-III-infected cells shares antigenic determinants with the 20-kDa protein synthesized in bacteria, the ability of the bacterial protein to compete in an immunoprecipitation reaction for the protein present in virus-infected cells was examined. Protein extracts prepared from bacterial cells that contain the pAS1-ART1 plasmid specifically competed for the 20-kDa polypeptide present in the infected cells (Fig. 4). The remaining HTLV-III-encoded proteins are not affected by the presence of the bacterial extract in the immunoprecipitation reaction. An extract prepared from bacteria that contain the out-of-frame plasmid, pAS1-ART3, was unable to compete for the 20-kDa polypeptide.

To confirm the identity of the 20-kDa protein, a synthetic peptide (Arg-Arg-Asn-Arg-Arg-Arg-Arg-Trp-Arg-Glu-Arg-Gln-Arg-Gln) corresponding to the nucleotide sequence at position 7993 to 8039 (according to the numbering scheme of Ratner et al. [14]) in the second coding exon of the *art* gene was synthesized and conjugated to keyhole limpet hemocyanin. Antibodies to the oligopeptide were raised in rabbits. The antibodies raised against the synthetic peptide also recognized a 20-kDa protein in H9 cells infected with HTLV-III (Fig. 5). A 20-kDa protein was not recognized by this serum in uninfected H9 cells nor was the 20-kDa protein immunoprecipitated by preimmune rabbit serum.

These experiments demonstrate that the *art* gene encodes a 20-kDa protein that is expressed in infected cells. Evidently, the *art* gene product is produced in the course of natural infection, as sera from some patients contain antibodies that react with the protein. The *art* open reading frame is capable of encoding a protein of 116 amino acids. The *art* protein, either as produced in *E. coli* or as expressed

in virus-infected cells, appears to migrate on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with an apparent molecular size of about 20 kDa, somewhat larger than the calculated size of about 13 kDa. The slower than anticipated electrophoretic mobility might be a consequence of the high content of charged amino acids (approximately 30% charged amino acids), or it could be due to unusual structural characteristics of the protein. It is noteworthy that the *tat* gene product of HTLV-III also migrates more slowly than anticipated.

Both the *tat*III and the *art* genes positively regulate viral *gag* and *env* gene expression and are necessary for HTLV-III replication. High-level production of the *art* gene protein should allow the purification of the protein as well as the production of monospecific anti-*art* antisera for further func-

FIG. 3. Protein expression by the pAS1-ART clones. To monitor protein expression, bacterial plasmids were retransformed into N99c1^{ts857}. Cells were grown at 30°C in minimal medium M56 supplemented with 0.2% glucose, 0.2 mM amino acid mixture, thiamine (1 µg/ml), and ampicillin (50 µg/ml) until the optical density at 650 nm equalled 0.3. A sample (0.2 ml) of these cells was incubated in the above medium lacking arginine at 30 or 42°C. After pulse-labeling, samples were either suspended directly in SDS-sample buffer or processed for immunoprecipitation. For immunoprecipitation, labeled cells were lysed with 50 µl of 1% SDS-1 mM EDTA and boiled for 2 min. A 650-µl portion of TSET (50 mM Tris hydrochloride, 150 mM NaCl, 0.1 mM EDTA, and 2% Triton X-100) was added and clarified. Lysates were then incubated with 100 µl of 10% protein A-Sepharose beads (Pharmacia) and 2 µl of serum from an AIDS patient overnight. Immunoprecipitates were washed twice with TSET and once with 10 mM Tris hydrochloride, pH 8.0, before being loaded on an SDS-polyacrylamide gel. Lanes: 1, 3, and 5, total bacterial extracts from pAS1-ART1, pAS1-ART2, and pAS1-ART3, respectively, pulse-labeled at 30°C; 2, 4, and 6, pAS1-ART1, pAS1-ART2, and pAS1-ART3 extracts, respectively, pulse-labeled at 42°C; 7, 9, and 11, immunoprecipitates with serum from an AIDS patient, RV119, of pAS1-ART1, pAS1-ART2, and pAS1-ART3, respectively, labeled at 30°C; 8, 10, and 12, immunoprecipitates with RV119 of pAS1-ART1, pAS1-ART2, and pAS1-ART3, respectively, induced at 42°C. Shown on the far left are ¹⁴C-labeled protein marker positions (Amersham Corp.).

FIG. 4. Immunocompetition of H9/HTLV-III cells with pAS1-ART bacterial extracts. About 2×10^6 H9 cells infected with HTLV-III were labeled for 12 h with [35 S]cysteine (100 μ Ci/ml) and [3 H]arginine (200 μ Ci/ml) for each immunoprecipitation reaction. Cells were lysed in 500 μ l of RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris hydrochloride [pH 7.2], 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) and clarified at $100,000 \times g$ for 1 h at 4°C . Total cellular lysates were then incubated with 5 μ l of serum from a patient and 80 μ l of 10% protein A-Sepharose beads (Pharmacia) overnight. Precipitated proteins bound to beads were washed six times with RIPA lysis buffer minus sodium deoxycholate and twice with 0.15 M NaCl-0.05 M Tris hydrochloride (pH 7.2) before being loaded on an SDS-polyacrylamide gel. Lanes: 1 and 2, immunoprecipitation reactions of H9/HTLV-III and H9 cells, respectively, with RV119, an AIDS patient serum; 3, 4, and 5, immunoprecipitates of H9/HTLV-III cells competed with 50, 150, and 200 μ g, respectively, of extracts of pAS1-ART1 bacteria induced at 42°C for 30 min; 6, 7, and 8, immunoprecipitates of H9/HTLV-III lymphocytes competed with 50, 150, and 200 μ g, respectively, of extracts of pAS1-ART3 bacteria induced at 42°C for 30 min.

tional studies. Antibodies to the *art* gene product may also serve as a useful indication of disease status.

We thank Martin Rosenberg of Smith Kline Beckman Corp. for providing the pAS1 vector and bacterial strains required for the work; J. Groopman, M. Essex, M. F. McLane, T. H. Lee, David D. Ho, and Martin Hirsch for discussion and materials; and Suresh Arya, Steven Josephs, Flossie Wong-Staal, and R. C. Gallo for providing the cDNA clone.

This work was supported by grants from the State of Massachusetts (W.A.H.) and by Public Health Service grant CA 07580 and contract FOD-0757 (C.A.R.), grants CA 40658 and CA 40659 (J.G.S.), and grant CA 42098 (W.A.H.) from the National Institutes of Health. J.G.S. and C.A.R. are Special Fellows of the Leukemia Society of America and are recipients of grants from the American Foundation for AIDS Research.

FIG. 5. Immunoprecipitation of H9/HTLV-III cells with antibodies against an *art* synthetic peptide. Lymphocytes were labeled and processed for immunoprecipitation as described in the legend to Fig. 4. Lanes: 1, an immunoprecipitation profile of infected cells with RV119, an AIDS patient serum; 2 and 3, H9/HTLV-III and H9 cells, respectively, precipitated with rabbit antibodies against a synthetic peptide corresponding to the *art* region as described in the text; 4 and 5, nonimmune rabbit serum with HTLV-III-infected and uninfected H9 cells, respectively.

LITERATURE CITED

1. Allan, J. S., J. E. Coligan, T. H. Lee, M. F. McLane, P. J. Kanki, J. E. Groopman, and M. Essex. 1985. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. *Science* **230**:810-813.
2. Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal. 1985. *Trans*-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science* **229**:69-73.
3. Barre-Sinoussi, F., J. C. Chermann, R. Rey, M. T. Nugeyre, S. Chanaret, J. Gruest, C. Daugey, C. Axler-Blin, F. Brun-Vezinet, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immunodeficiency syndrome (AIDS). *Science* **220**:868-871.
4. Dayton, A. I., J. G. Sodroski, C. A. Rosen, W. C. Goh, and W. A. Haseltine. 1986. The *trans*-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* **44**:941-947.
5. Fisher, A. G., M. B. Feinberg, S. F. Josephs, M. E. Harper, L. M. Marselle, G. Reyes, M. A. Gonda, A. Aldovini, C. Debouk, R. C. Gallo, and F. Wong-Staal. 1986. The *trans*-activator gene of HTLV-III is essential for virus replication. *Nature (London)* **320**:367-371.
6. Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, and P. D. Markham. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**:500-503.
7. Goh, W. C., C. Rosen, J. Sodroski, D. D. Ho, and W. A. Haseltine. 1986. Identification of a protein encoded by the *trans* activator gene *tatIII* of human T-cell lymphotropic retrovirus type III. *J. Virol.* **59**:181-184.
8. Gottlieb, M. S., R. Schroff, H. M. Schenker, J. D. Weisman, P. T. Fan, R. A. Wolf, and A. Saxon. 1981. *Pneumocystis carinii* pneumonia and mucosal carinii pneumonia: initial manifestation of cellular immune dysfunction. *N. Engl. J. Med.* **305**:1425-1431.
9. Kan, N. C., G. Franchini, F. Wong-Staal, G. C. DuBois, W. G.

- Robey, J. A. Lautenberger, and T. S. Papas. 1986. Identification of HTLV-III/LAV *sor* gene product and detection of antibodies in human sera. *Science* 231:1553-1555.
10. Lane, H. C., H. Masue, L. C. Edgar, G. Whalen, A. H. Rook, and A. S. Fauci. 1984. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 309:453-458.
11. Lee, T. H., J. E. Coligan, J. S. Allan, M. F. McLane, J. E. Groopman, and M. Essex. 1986. A new HTLV-III/LAV protein encoded by a gene found in cytopathic viruses. *Science* 231:1546-1549.
12. Levy, J. A., A. D. Hoffman, S. M. Kraamer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* 225:840-842.
13. Masur, H., M. A. Michelis, J. G. Greene, I. Onorato, R. A. Vande Stouwe, R. S. Holzman, G. Wormser, L. Brettman, M. Lange, H. W. Murray, and S. Cunningham-Rundles. 1981. An outbreak of community-acquired *Pneumocystis carinii* pneumonia: initial manifestation of cellular immune dysfunction. *N. Engl. J. Med.* 305:1431-1438.
14. Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Bauneister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* 313:277-284.
15. Rosenberg, M., Y. S. Ho, and A. Shatzman. 1983. The use of pKC30 and its derivatives for controlled expression of genes. *Methods Enzymol.* 101:123-139.
16. Sarngadharan, M. D., M. Popovic, L. Bruch, J. Schupach, and R. C. Gallo. 1984. Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science* 224:506-508.
17. Shaw, G. M., M. E. Harper, B. H. Hahn, L. G. Epstein, D. C. Gajdusek, R. W. Price, B. A. Navia, C. K. Petito, C. J. O'Hara, J. E. Groopman, E. S. Cho, J. M. Oleske, F. Wong-Staal, and R. C. Gallo. 1985. HTLV-III infection in brains of children and adults with AIDS encephalopathy. *Science* 227:177-181.
18. Siegal, F. P., C. Lopez, G. S. Hammer, A. E. Brown, S. J. Kornfield, J. Gold, J. Hassett, S. Z. Hirshman, C. Cunningham-Rundles, B. R. Adelsberg, D. M. Parham, M. Siegal, S. Cunningham-Rundles, and D. Armstrong. 1981. Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions. *N. Engl. J. Med.* 305:1439-1444.
19. Sodroski, J., W. C. Goh, C. Rosen, A. Dayton, E. Terwilliger, and W. Haseltine. 1986. A second post-transcriptional *trans*-activator gene required for HTLV-III replication. *Nature (London)* 321:412-417.
20. Sodroski, J., W. C. Goh, C. Rosen, A. Tartar, D. Portetelle, A. Burny, and W. Haseltine. 1986. Replicative and cytopathic potential of HTLV-III/LAV with *sor* gene deletions. *Science* 231:1549-1553.