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Subcellular Localization of the Human Immunodeficiency Virus *trans*-Acting *art* Gene Product

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The genome of the human immunodeficiency virus is distinguished from other animal retroviruses by the presence of several additional open reading frames. The protein product of one of these novel genes, which has been termed *art* or *trs*, is required for the expression of the virus structural genes but not for the expression of virus encoded regulatory proteins. Immunocytochemistry and subcellular fractionation demonstrate that the *art* protein is located predominantly in the nucleus. Therefore, any proposed mechanism for the function of *art* is likely to involve nuclear events.

The pathogenic human retrovirus human immunodeficiency virus (HIV) encodes the genes for several nonstructural proteins of which at least two, termed *tat* (1, 20) and *art* (21), are essential for virus replication (5). The HIV *tat* gene encodes a *trans*-acting nuclear protein (11) whose coexpression results in a large amplification of HIV gene expression (1, 20). The *tat* protein appears to act via a bimodal mechanism that results in the enhancement of both viral mRNA transcription and utilization (3, 13, 14, 17, 18, 23). Loss of *tat* function results in the loss of detectable viral gene expression (5, 7, 17). In contrast, loss of *art* function prevents only the expression of viral structural proteins, including *gag* and *env*, whereas expression of nonstructural proteins, including those encoded by *tat* and *3'orf*, remains unaffected (6, 12, 19). Therefore, the action of *art* would be predicted to allow the progression of the HIV replication cycle from the early (nonstructural protein) to the late (structural protein) phases observed for DNA viruses but not previously described for retroviruses. However, at present the mechanism of *art* action remains unclear.

One approach which may facilitate the understanding of how a protein functions is to determine where it functions. To address this question, we constructed expression vectors containing either a spliced *art* cDNA sequence (pH3-*art*, pSV2-*art*) or an unspliced, genomic version of the *art* gene (pBC12/CMV/*art*) (Fig. 1A). To demonstrate that the *art* proteins expressed from these vectors were functional, we used two indicator plasmids, termed pIIIAR (18a) and pSV-AR (Fig. 1B), that contain a segment of the HIV *env* gene inserted into (i) the 3′ noncoding region of the HIV long terminal repeat based chloramphenicol acetyl transferase (CAT) expression vector pU3R-III (20) and (ii) the simian virus 40 early promoter-based CAT expression vector pSV2-CAT (10). In a previous study with the pIIIAR vector (18a), we demonstrated that sequences located within the coding region of the *env* gene can inhibit the expression of heterologous genes when present in *cis*, and that the repressive effects of these sequences can be relieved by the coexpression in *trans* of the *art* protein. Thus, the expression of the CAT gene linked to these HIV proviral sequences mimics the regulation observed with the *gag* and *env* genes themselves and therefore permits the simple demonstration of *art* function (Fig. 1B). Both the HIV long terminal repeat (pIIIAR; Fig. 1B)- and simian virus 40 early promoter (pSV-AR)-based CAT constructions containing the inserted *env* sequences display very low levels of CAT activity after transfection of COS cells (Fig. 1C, lanes 1 and 4). However, cotransfection of pH3-*art* (Fig. 1C, lane 2), pSV2-*art* (Fig. 1C, lane 5), or pBC12/CMV/*art* (Fig. 1C, lanes 3 and 6) resulted in a large increase in CAT expression.

The generation of a polyclonal rabbit antibody directed against a synthetic *art* peptide derived from amino acids 38 to 51 of the 116-amino-acid *art* open reading frame (9) (here termed antibody Art38/51) was previously described. Two additional rabbit polyclonal antibodies directed against synthetic peptides derived from amino acids 1 to 20 (Art1/20) and amino acids 27 to 51 (Art27/51) were prepared. Antibody Art1/20 was directed against a protein sequence encoded by the first *art* gene coding exon, whereas antibodies Art27/51 and Art38/51 were directed against overlapping sequences derived from the second *art* coding exon (Fig. 1). Each antibody was able to specifically immunoprecipitate a 20-kilodalton protein from COS cells transfected with either the genomic *art* expression vector pBC12/CMV/*art* or the cDNA expression vector pSV-*art* (Fig. 2). No specific signal was detected in COS cells transfected with a *tat* expression vector or with pBC12/CMV/*art* transfected cells probed with preimmune rabbit serum (Fig. 2).

Immunocytochemistry and cell fractionation were used to determine the intracellular location of the *art* gene product. The Art1/20 and Art27/51 antisera were first used to localize the *art* protein within COS cells transfected with the pBC12/CMV/*art* expression vector by indirect immunofluorescence

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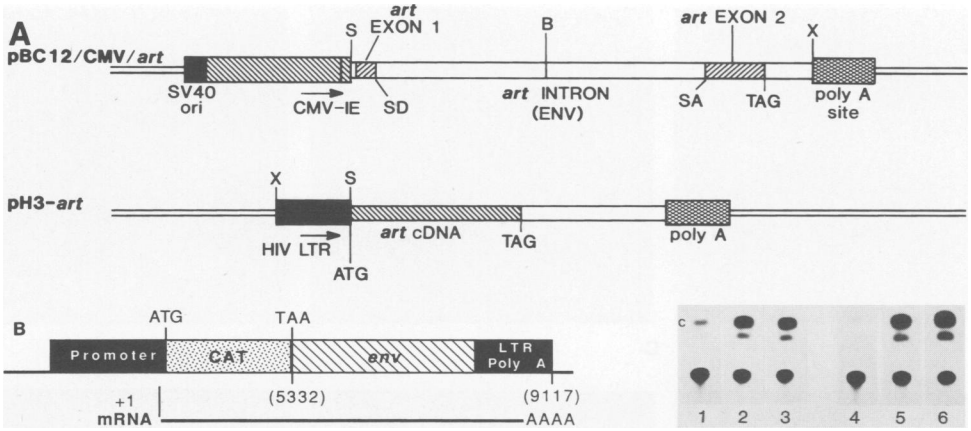

FIG. 1. Structural (A) and functional (B and C) analysis of the *art* expression and *art* indicator vectors. (A) pBC12/CMV/*art* encodes a genomic copy of the HIV *art* gene under control of the human cytomegalovirus immediate early (CMV-IE) promoter (3). In the HIV genome, the *art* open reading frame is divided into two coding exons separated by an intronic region derived from the viral *env* gene (21). A DNA fragment containing the entire genomic *art* gene was isolated after cleavage of the replication competent HXB-3 proviral HIV clone (15) at a 5′ *Sal*I site and a 3′ *Xho*I site followed by blunt ending with Klenow DNA polymerase I. The expression vector pBC12/CMV (3) was cleaved at a 5′ *Hin*dIII site and a 3′ *Bam*HI site and also blunt ended. The insertion of the HIV DNA fragment into the pB12/CMV vector in the sense orientation results in the restoration of the 5′ *Hin*dIII site and of the 3′ *Xho*I site. Subsequently, this intermediate plasmid construction was cleaved with *Hin*dIII and *Mst*II and religated after insertion of a *Sal*I linker molecular (5′-GGTCGACC-3′). This results in the deletion of a 169-base-pair HIV DNA fragment which includes the *tat* gene translation initiation codon and instead aligns the *art* initiation codon with the 5′ end of the mRNA encoded by the pBC12/CMV/*art* vector. The construction of the *art* cDNA expression vector pH3-*art* has been previously described (18a). Plasmid pSV2-*art* is identical to pH3-*art* except that the HIV long terminal repeat sequence has been replaced with the simian virus 40 early region promoter obtained from plasmid pSV2CAT (10). SD, Splice donor; SA, splice acceptor; ATG, *art* translation initiation codon; TAG, art translation termination codon; S, *Sal*I; X, *Xho*I; B, *Bgl*II. (B) Plasmids pIIIAR and pSV-AR contain HIV *env* sequences (nucleotides 5332 to 9177) inserted into the 3′ noncoding portion of the CAT gene transcript. Transcriptional control sequences for plasmids pIIIAR and pSV-AR were derived from HIV long terminal repeat sequences −167 to +80 and simian virus 40 early region promoter sequences, respectively. A more detailed description on the use of these plasmids is given elsewhere (18a). (C) Effect of the *art* gene product on CAT gene expression directed by hybrid HIV/CAT plasmids. COS cells were transfected (4) with 1 μg of each of the indicated plasmid DNAs, and CAT assays (10) were performed 48 h posttransfection. The COS cells transfected with pIIIAR (lanes 1 through 3) were also cotransfected with 1 μg of the *tat* expression vector pD(83-5365/053-9296) (1). Lanes contained COS cells transfected with the following: 1, pIIIAR and pSV2NEO (21); 2, pIIIAR plus pH3-*art*; 3, pIIIAR plus BC12/CMV/*art*; 4, pSV-AR plus pSV2NEO (22); 5, pSV-AR plus pSV2-*art*; 6, pSV-AR plus pBC12/CMV/*art*.

microscopy. The *art* protein was predominantly located within the nucleus of expressing cells (Fig. 3). The distribution within the nucleus appeared nonrandom, since both the Art1/20 and Art27/51 antibodies reveal similar patterns of intense and weak nuclear staining. No fluorescence was observed when COS cells were transfected with a vector lacking *art* sequences or when preimmune serum was used (data not shown).

In a second set of experiments, the intracellular location of *art* was determined by using subcellular fractionation techniques. We have described the development of HeLa and CHO cell lines which stably express the HIV *tat* gene product after infection with an amphotropic retroviral *tat* expression vector (16). These tat^+ cells were cotransfected with plasmids pH3-*art* and pSV2-gpt (21), which confer resistance to the drug mycophenolic acid. Functional constitutive expression of the *art* gene product by the individual mycophenolic acid-resistant HeLa and CHO clonally derived cell lines was confirmed by measurement of CAT gene expression after transfection with the pIIIAR indicator construction (data not shown). To identify the subcellular location of the *art* protein, a clonal HeLa tat^+ art^+ cell line and two CHO tat^+ art^+ clonal cell lines showing different levels of constitutive *art* protein expression were fractionated into nuclear and membrane-cytoplasmic fractions (8). Proteins present in the individual fractions were then separated by electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted with the rabbit antibody Art38/51 (Fig.

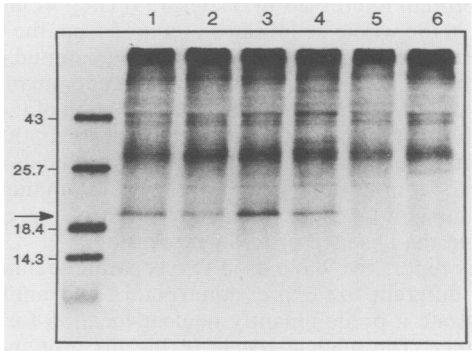

FIG. 2. Immunoprecipitation analysis of *art* protein synthesized in transfected COS cells. COS cells were transfected (4) with the indicated plasmids and pulsed-labeled for 2 h with [^{35}S]cysteine at 48 h posttransfection. Immunoprecipitation of labeled *art* protein was performed as previously described (4) with a 1:500 dilution of the indicated anti-*art* antibody. All three antibodies were raised in rabbits by injection of each synthetic *art* peptide conjugated to keyhole limpet hemocyanin and suspended in Freund complete adjuvant. The negative control *tat* gene expression vector used in lane 6, pBC12/CMV/t2, has been previously described (3). Lanes contained COS cells transfected with the following: 1 pBC12/CMV/*art* and antibody Art27/51; 2, pBC12/CMV/*art* and Art1/20; 3, pSV2-*art* and Art27/51; 4, pBC12/CMV/*art* and Art38/51; 5, pBC12/CMV/*art* and preimmune antiserum from the rabbit used to generate the Art27/51 antibody; 6, pBC12/CMV/t2 and Art27/51.

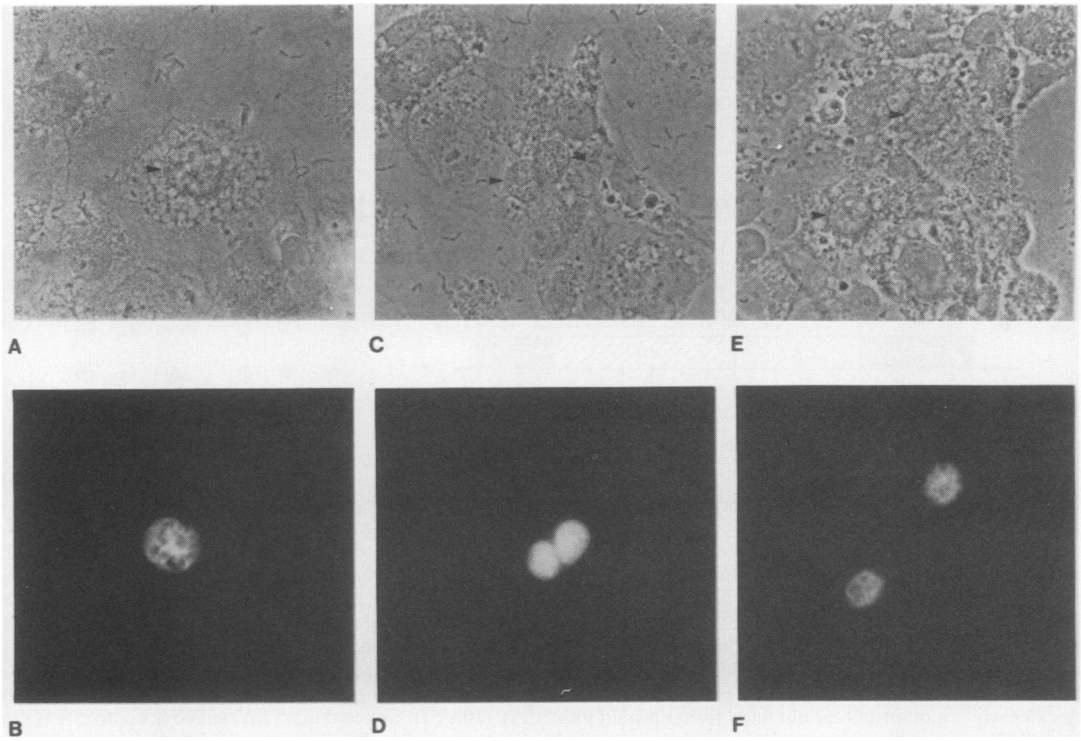

FIG. 3. Subcellular localization of *art* protein within transfected COS cells by indirect immunofluorescence. Phase-contrast and corresponding immunofluorescence photographs of COS cells transfected with pBC12/CMV/*art*. The cells were fixed and permeabilized (11) at 72 h after transfection and then treated with a 1:800 dilution of rabbit polyclonal anti-peptide Art1/20 (A and B) or Art27/51 (C through F) antibodies followed by 1:50 dilution of rhodamine conjugated goat anti-rabbit antibody (Boehringer Mannheim Biochemicals). The photograph in panel D was intentionally overexposed to illustrate the absence of any detectable cytoplasmic fluorescence in cells expressing *art*. Nuclear *art* expression was detected in 5 to 10% of the transfected cells, the percentage expected to have incorporated plasmid DNA as a result of our transfection procedure (4). Note the areas of intense and weak nuclear staining visualized in panels B and F.

4). A 20-kilodalton protein that comigrated with the authentic *art* protein expressed in bacteria (9) (Fig. 4, lane 2) was evident in the whole cell lysates prepared from the CHO and HeLa *tat art* clones but was absent in the parental CHO and HeLa cells. This protein was present predominantly in the nuclear fraction. The subcellular fractionation of these HeLa and CHO cell clones, which stably express both the HIV *trans*-acting proteins *tat* and *art*, therefore yields the same nuclear location for *art* as that observed by immunofluorescent staining of COS cells, which transiently express the *art* protein in the absence of *tat* coexpression.

In this report, we have used two separate techniques and several different *art* expression vectors and antibodies to demonstrate a predominantly nuclear location for the HIV *art* protein. The nuclear locale of the *art* protein provides clues regarding the mechanism of action of this protein. All viral proteins are translated from mRNAs derived from the same primary transcript, some from the full-length genomic transcript and some from spliced products. However, individual viral proteins are affected differently by the presence of the *art* gene product, thus indicating that the *art* protein must act to regulate protein expression posttranscriptionally. The nuclear location of the *art* gene product suggests that this protein does not interact directly with the translational machinery but rather regulates protein expression by affecting the fate of mRNA in the nucleus. This posttranscriptional regulation of gene expression by the nuclear *art* protein could occur by several different mechanisms, including determination of the nuclear half-life of the primary

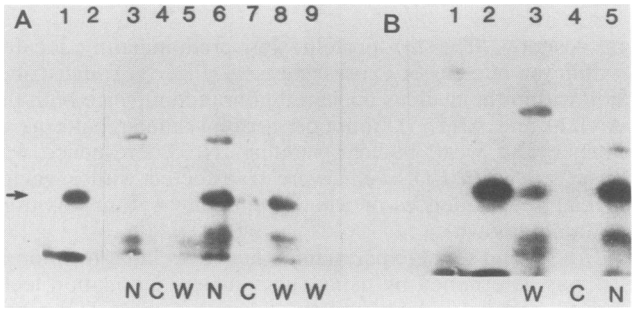

FIG. 4. Immunoblot analysis of subcellular fractions of cells expressing *art*. The CHO and HeLa clonal lines used for fractionation were phenotypically art^+ and tat^+. Cells were fractionated into nuclear and membrane-cytoplasmic fractions by treatment with nonionic detergent in the presence of an isoosmotic solution under conditions that stabilize the nuclear membrane (8). Nuclei were separated from other cellular components by differential centrifugation. Nuclear fractions were judged greater than 95% pure as determined by measurement of levels of the lysosomal enzyme β-*N*-acetyl glucosaminidase (2) (data not shown). Nuclear fractions (N), membrane-cytoplasmic fractions (C), and whole cell lysates (W) were analyzed by immunoblotting (Promega Biotech, Inc., Madison, Wis.) with the rabbit polyclonal anti-peptide antibody Art38/51. (A) Lysates prepared from control *Escherichia coli* cells (lane 1) or *E. coli* that overexpress *art* (9) (lane 2), CHO *tat art* clone 5 cells (lanes 3, 4, and 5), and CHO *tat art* clone 1 cells (lanes 6, 7, and 8), or CHO cells (lane 9). (B) Lysates prepared from control *E. coli* (lane 1), *E. coli* that overexpress *art* (lane 2), or HeLa *tat/art* cells (lanes 3, 4, and 5).

transcript and spliced products, regulation of splicing of the primary transcript as has been suggested (6), or regulation of mRNA transport from the nucleus to the cytoplasm. Interestingly, these proposals, are, in part, similar to the mechanisms that have been suggested for the posttranscriptional component of the bimodal mechanism of action of *tat*, the second *trans*-acting nuclear protein encoded by HIV (11). This raises the possibility that *art* and *tat*, which appear to be encoded by a single dicistronic mRNA (19), could functionally interact to modulate the level and character of HIV-specific gene expression. This intriguing possibility should clearly be considered in further experiments designed to address the molecular nature of *art* and *tat* function.

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