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ONCOGENE ELEMENTS WITHIN AN ENDOGENOUS RETROVIRUS

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Summary. – The human genome contains a large number of endogenous retroviral-related sequences. While the function of these sequences is unknown, they may contribute to disease processes through their regions of homology with infectious retroviruses. We have been further characterizing a recently reported HTLV-1 related endogenous retroviral sequence cloned from T lymphocytes isolated from a patient with essential cryoglobulinemia. We here report further detailed transcriptional analysis of the sequence for tissue and cell-cycle specificity and a novel finding of an association between the endogenous retrovirus and a *ras*-related gene.

Key words: endogenous retrovirus; autoantibody; oncogene autoimmune disease

Introduction

Infectious retroviruses contain the entire viral genome necessary to traverse the viral life cycle (Varmus, 1988). These infectious retroviruses have germline counterparts termed endogenous retroviruses (ERVs), which are defective retroviruses that lack one or more genes necessary for replication. ERVs have been identified and characterized based on their regions of homology to different infectious retroviruses. These endogenous sequences are inherited in a Mendelian fashion and are thought to comprise at least 10 % of human chromosomal DNA (Abraham and Khan, 1990). In addition to vertical transmission, the sequences may have become dispersed throughout a recently identified process of amplification of the ERV DNA and its flanking regions (Steele *et al.*, 1986).

Evidence has been accumulating to implicate ERVs in autoimmune disease processes through mechanism based on their similarities to infectious retroviruses. Several investigations have implicated endogenous retroviral proteins as stimuli for the genesis of autoimmune responses. Studies have demonstrated antibodies that cross-react with endogenous viral proteins and self-components in the serum or tissues of patients with autoimmune diseases, including Sjogren's Syndrome (SS), multiple sclerosis (MS) (Brookes *et al.*, 1991; Talal *et*

al., 1990*a*), mixed connective tissue diseases (MCTD) (Rucheton *et al.*, 1985), systemic lupus erythematosus (SLE) (Talal *et al.*, 1990*b*; Phillips *et al.*, 1986), and cryoglobulinemia (Perl *et al.*, 1991). In SS and SLE patients, serum antibodies that cross react with proteins from HIV-1 have been identified; in MS and cryoglobulinemia, antibodies cross reacting with HTLV-1 proteins have been identified; and in MCTD, antibodies against murine leukemia virus have been demonstrated. Supporting evidence for a causal relationship between the viral proteins and autoantibodies was obtained in a mouse model in which an autoantibody reactive with anti-U1 RNP could be generated by immunization with a retroviral *gag* protein (Query and Keene, 1987).

In addition to providing potential antigenic determinants to which a cross-reactive autoimmune response could be generated, retroviral sequences may influence the immune system through direct effects of their proteins. This mechanism is demonstrated by the retroviral envelope protein termed p15E, which has been shown to exert direct immunosuppressive effects by inhibiting stimulation of lymphocytes (Hebebrand *et al.*, 1979; Cianciolo *et al.*, 1985; Mitani *et al.*, 1987). An endogenous retroviral sequence homologous to p15E has also been identified and appears to produce similar immunosuppression (Krieg *et al.*, 1989*a*).

To investigate potential roles for ERVs in autoimmune disease, Perl *et al.* (1989) recently identified and isolated a new ERV (HRES-1/1) that was present in the DNA of activated T cells obtained from a patient with Type II cryoglobulinemia. This defective retrovirus contains an apparently intact long terminal repeat and viral elements that are homologous to segments of the *gag* region of HTLV-1 (Perl *et al.*, 1989). The goals of the present study were to further characterize HRES-1/1 in terms of genomic representation and polymorphism and to assess potential functions of the ERV through an investigation of its transcriptional activity in different tissues. We here report that HRES-1/1 locus is transcriptionally active in a tissue specific manner, transcriptional activity is not cell-cycle dependent, there is a second polymorphism associated with the locus, and the locus has a striking homology with a ras-related gene, suggesting a new function for this HRES-1/1 in oncogene transmission.

Materials and Methods

Cell lines. All cell lines were obtained from the American Type Culture Colection (except for HL-60 lines which were a gift of Dr. C. Abboud, University of Rochester and MA-T cells, which were isolated from a patient with cryoglobulinemia) and maintained in either DMEM or RPMI medium (Sigma), as recommended by the ATCC. Human cell lines reported in the study are as follows. FHs738Lu (normal foetal lung), Hs294T (melanoma), Hs638 (glioma), CaOv-3 (ovarian carcinoma), Kato III (gastric carcinoma), K562 (chronic myelogenous leukemia), Jurkat (T-cell leukemia), Daudi (Burkitt's lymphoma), NCI-H69 (lung-small cell carcinoma), Colo-320 (colon adenocarcinoma), Hs67 (normal thymus), and JAR (placental choriocarcinoma).

Southern blot analysis. DNA was isolated from cells by standard methods (Sambrook *et al.*, 1989). 10μg of genomic DNA isolated from cell lines was digested with either *Hind*III or *Eco*RI and

electrophoresed in a 0.9 % agarose gel, which was then blotted onto Zetabind (Bio-Rad). The membranes were baked at 80 °C for 1 hr and pre-hybridized at 42 °C for at least 1 hr in 5 × SSPE and 50 % formamide. Probes were labelled by random prime labelling with ^{32}P-dCTP (NEN), 10^6 cpm/ml were added to membranes in the pre-hybridization fluid, and the solution was incubated overnight at 42 °C. Blots were washed at high stringency (65 °C, 0.1 ×SSC, 0.1 % SDS for 90 min). Membranes were exposed to standard X-ray film (Kodak) overnight in the presence of Cronex II intensifying screens and developed.

Northern blot analysis. RNA was isolated and Northern blot analysis was performed according to standard methods (Sambrook *et al.*, 1989). RNA was electrophoresed in 1.5 % agarose/formaldehyde gels and blotted onto nitrocellulose (Schleicher and Schuell). Probes were generated by cloning restriction fragments of the HRES-1/1 locus spanning positions 927-1357 or positions 1323-1609, into pBluescript (Stratagene). Single stranded RNA probes were transcribed with either T7 or T3 primers, to generate probes that are complementary to the coding sequence and have opposite orientation.

Cell-cycle separation. Epstein-Barr virus transformed B cells from a patient with cryoglobulinemia were selected for cell-cycle analysis. Centrifugal elutriation was performed as previously described in detail (Palis *et al.*, 1988). Separation was effected based on DNA and RNA content and cell size, since these parameters increase as cells progress through the cycle. Cell fractions containing 90 % G_1 cells, > 85 % S cells, and > 75 % G_2-M cells were collected and RNA was immediately isolated.

Computer analysis. The HRES-1/1 sequence has been entered into the GenBank, accession number X16660. FastA searches (Pearson and Lipman, 1988) were executed with the entire sequence and with individual regions to identify other sequences with areas of homology.

Results

Transcriptional activity

1. Tissue specificity

We have found that the HRES-1/1 locus is transcriptionally active (Fig. 1, 2). Transcripts of 5.5 kb were detected in the following human cell lines that have not been previously reported: melanoma, normal thymus, normal foetal lung, lung cell carcinoma, HL-60 cells of three lineages, an ovarian carcinoma.

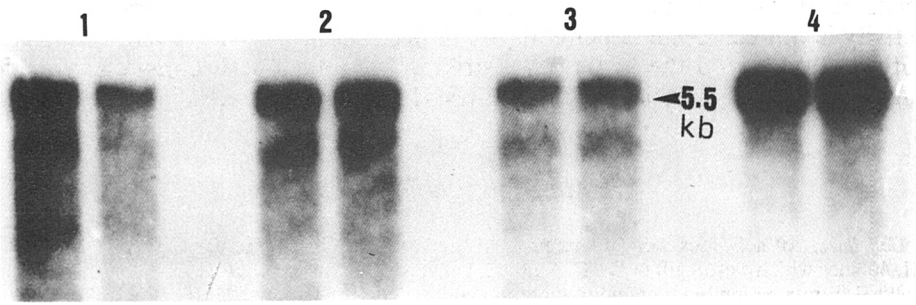

Fig. 1

Northern blot analysis of RNA

Cell lines analyzed: CaOv (1), K562 (2), HL-60 (3) and U937 (4). 5 μg of RNA was electrophoresed in agarose/formaldehyde gel, transferred to nitrocellulose and probed with a riboprobe complementary to the coding strand of HRES-1/1.

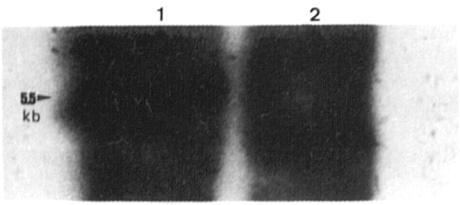

Fig. 2
Northern blot analysis of RNA
Cell lines analyzed: NCI-H69 (1) and Hs638 (2).

a placental choriocarcinoma and Epstein-Barr virus (EBV) transformed B cells from healthy volunteers. Transcripts were not detected in cells from a glioma, or from gastric or colon carcinomas. RNA integrity was ensured by probing with actin (not all data shown).

2. Association with polymorphism

A *Hind*III polymorphism was previously reported in the HRES-1/1 locus. We have noted an additional polymorphism in the HL-60 cell lines with *Eco*RI (Fig. 3). Testing of cells of previously defined genotypes revealed that this polymorphism did not appear either to be linked to the *Hind*III polymorphism or to be associated with transcriptional activity.

3. Association with cell-cycle

To determine whether transcriptional activity was cell-cycle dependent, EBV-transformed B cells were separated into fractions enriched for cells in G_1, S, and

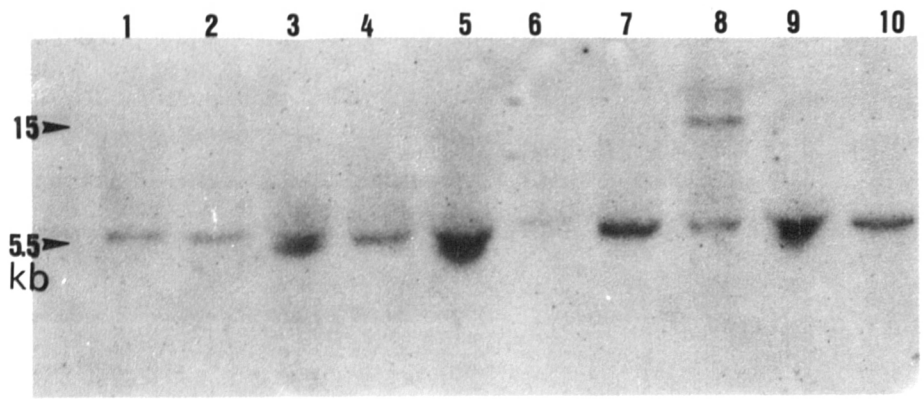

Fig. 3
Southern blot analysis of DNA
Cell lines analyzed: Daudi (1), Colo-320 (2), Hs738lu (3), Hs294T (4), Hs638 (5), U937 (6), K562 (7), HL-60 (8), Kato-111 (9) and CaOv (10). 10 μg of DNA was digested with *Eco*RI, electrophoresed in agarose gel, transferred to Nytran and probed with the *Sma-Hind*III restriction fragment of HRES-1/1 (nt 927–2147).

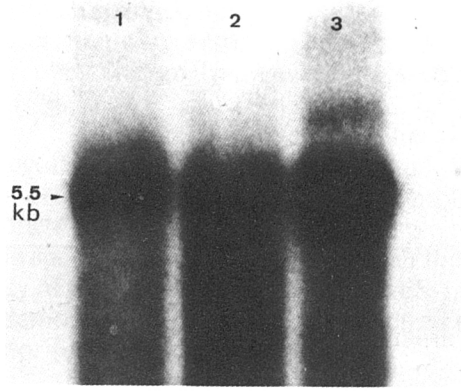

Fig. 4
Northern blot analysis of RNA from cell-cycle sorted EBV-transformed B cells from a patient with cryoglobulinemia
Cells were analyzed in phase G_1 (lane 1), S (lane 2) and G_2-M (lane 3).

G_2-M. No difference in transcriptional activity was noted for cells at different phases of the cell-cycle, as a transcript was present for each fraction (Fig. 4).

Association with a ras-related gene

To identify other potentially functional areas of the locus, further homology searches were performed with individual regions of the sequence. In addition to its similarities to viruses, HRES-1/1 also shares a region of striking homology with the *ras*-related gene, *rab* (Zahraoui *et al.*, 1989). The sequences are 97 % homologous over 88 base pairs (Fig. 5, 6). Since only one band had previously been detected in Southern blot analysis, we sought to investigate the relationship between these two genes. To determine whether the *rab* gene was present on the same restriction fragment as the HRES-1/1 a shorter probe was selected that spanned positions 1323-1609, which shared a larger region of homology to the

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ERV (BASE 1454)  C G G A C C G C G G G C G A G T - G C A C G G T
                 I I I I I I I I I I I I I I I I I I I I I I I I
RAB (BASE 1)     C G G A C C G C G G G C G A G T G G C A C G G T

G A C C C G G C G A G A G G C G G C G C C G C T C C C A A G A T G
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I I I I I
G A C C C G G C G A G A G G C G G C G C C G C T C C C A A G A T G

T C G C A G A C G G C C - A T G T C C G A A A C C T A C G G T A
I I I I I I I I I I I I   I I I I I I I I I I I I I I I I I   I
T C G C A G A C G G C C A A T G T C C G A A A C C T A C G A T T
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Fig. 5
Homology between HRES-1/1 and *rab*

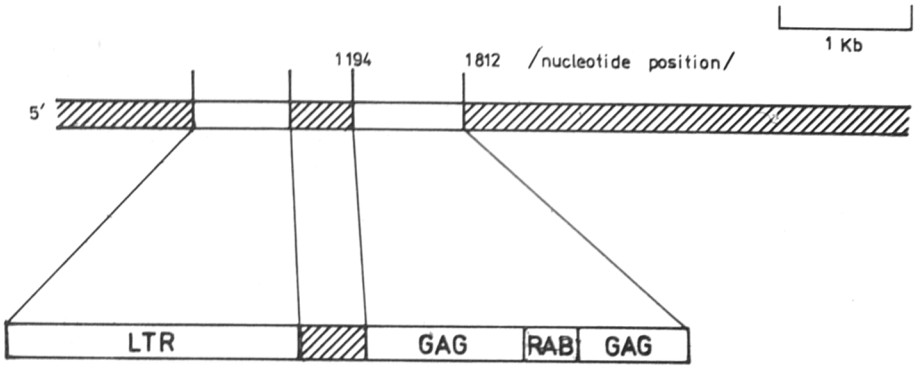

Fig. 6
Schematic diagram of the relationship between HRES-1/1 and *rab*

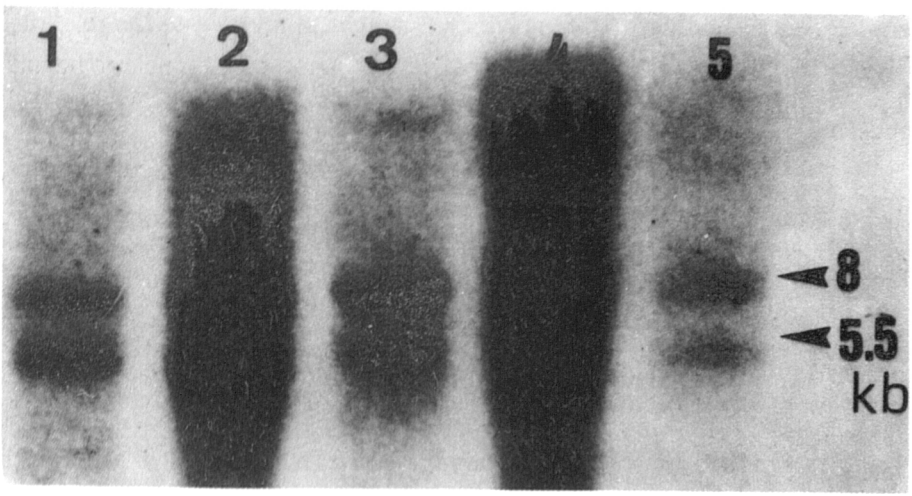

Fig. 7
Southern blot analysis of DNA
Cell lines analyzed: CaOv (1), HL-60 (2), Daudi (3), Hs738lu (4) and Hs638 (5). Blots were probed with the restriction fragment spanning nt 1323–1609.

rab gene than the previously used probe, which spanned positions 927–2147. The Southern blots described in the above experiments were stripped of the previous probe and re-hybridized with the new probe. This analysis revealed a second fragment present in all cell lines tested (Fig. 7).

The homology between HRES-1/1 and the *rab* is of an inverse nature: the coding strand of the HRES-1/1 is homologous to the anti-sense strand of *rab*. To determine whether the inverse strand of HRES-1/1 was transcribed, the probe was cloned into pBluescript and transcribed with the T7 primer to generate an RNA probe. Northern blot analysis was repeated with RNA from the cell lines reported above. No transcriptional activity of the inverse strand of the HRES-1/1 was detected. Activity of the probe was demonstrated as the probe hybridized to the DNA sequence cloned into the vector (data not shown).

Discussion

We have further characterized a novel HTLV-1-related endogenous retroviral sequence cloned from T cells isolated from a patient with the benign monoclonal gammopathy, cryoglobulinemia. To clarify roles for this sequence, we have evaluated HRES-1/1 in terms of genomic representation and differential transcriptional activity. In addition, we also report a novel potential function of the HRES-1/1 in transmission of an oncogene.

Southern blot analysis of genomic representation revealed that the locus was present in all human cell lines tested. A previously reported *Hind*III polymorphism was confirmed in these studies, and an additional *Eco*RI polymorphism was noted in the HL-60 cell line. The polymorphisms did not appear to be linked to each other, and neither appeared to affect the transcriptional activity of the locus.

Northern blot analysis revealed a differential expression of activity of the locus in that it was transcriptionally active in a tissue-specific manner. This transcription appeared to remain consistent throughout the cell-cycle. To our knowledge, no analysis of cell-cycle-associated expression of an ERV has been reported. Further, transcriptional activity did not appear to depend on the stage of development of the cells, since transcripts were present in foetal and adult tissue, or on the cell line being derived from a malignant transformation process, since a transcript was detected in both a normal lung and in a lung cell carcinoma.

The ERV contains a region that is homologous to the *gag* regions found in infectious retroviruses (Perl *et al.*, 1987), suggesting one mechanism by which the locus may be involved in pathogenesis of autoimmune disease in that expression of this *gag* region may lead to a cross-reactive autoimmune response. Antibodies reactive with this region of the ERV have been identified in patients with Sjogren's syndrome (Brookes *et al.*, 1991). Potential for this and other mechanisms has been evaluated in other systems. In a murine model of systemic lupus erythematosus, it was demonstrated that a full length (8.4 kb) transcript

was present in autoimmune-prone mice that was undetectable in control mice. Further, this differential expression was present prior to disease onset in the autoimmune mice. The hypothesis that these and other observations generate is that endogenous retroviral proteins may be displayed on the cell surface and when recognized by the immune system in the appropriate context could stimulate a self-perpetuating immune response (Krieg *et al.*, 1988; Krieg *et al.*, 1989*a, b*). This hypothesis has been supported by human studies in which cross-reactive antibodies have been found in patients with mixed connective tissue disorders (Rucheton *et al.*, 1985). Antibodies that react with a number of retroviral proteins have also been described in patients with cryoglobulinemia (Brookes *et al.*, 1991; Perl *et al.*, 1991).

In addition to the mechanisms described in the above studies, the homology between HRES-1/1 and *rab* suggests novel functions by which an endogenous sequence might contribute to pathology. One mechanism by which this homology may be significant in disease is by facilitating transmission of this *ras*-related gene or other oncogene elements. Since ERVs are thought to have become dispersed throughout the genome through amplification of the ERV and its flanking sequences, (Steele *et al.*, 1980), it is possible that an associated oncogene could fortuitously be translocated in the process and potentially activated. In addition, since the coding strand of this ERV is homologous to the anti-sense strand of the *ras*-related gene, the sequence could not have been acquired through reverse transcription, which suggests an unusual mechanism of oncogene element translocation.

A further function of ERVs has been proposed in murine studies on intracisternal A particles (IAPs), which are defective endogenous retrovirions. These studies suggest that the IAPs may contribute to neoplastic transformation of cells through activation of growth hormone genes or other cellular proto-oncogenes (Kuff, 1990). IAPs have also been detected in the salivary glands of patients with Sjogren's syndrome (Garry *et al.*, 1990).

A second potential mechanism of action related to the homology is through regulation. Action through regulation could include a competition for transcription factors, or a regulatory product encoded by one sequence affecting activation of the other, or that when one sequence is transcribed it activates (or represses) the other. Alternatively, perhaps there may be an association with the G protein function of the *ras*-related gene that could be relevant to the malignant transformation observed in cryoglobulinemia patients. Further investigation will be necessary to determine the role of this sequence in the pathogenesis of cryoglobulinemia.

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