Gene-expression changes induced by *Feline immunodeficiency virus* infection differ in epithelial cells and lymphocytes

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Infection of cats with *Feline immunodeficiency virus* (FIV) is an important model for understanding comparative lentivirus biology. *In vivo*, FIV infects lymphocytes and monocyte/macrophages, but *in vitro* infection is commonly investigated in epithelial Crandell–Reese Feline Kidney (CRFK) cells. In this study, the transcriptional responses of CRFK cells and primary lymphocytes to infection with FIV 34TF, a cloned subtype A virus, and FIV USgaB01, a biological subtype B isolate, were determined. Reverse-transcribed mRNA from both cell types was hybridized to microarrays containing 1700 human expressed sequence tags in duplicate and data were analysed with Significance Analysis of Microarrays (SAM) software. Results from six experiments assessing homeostatic cross-species hybridization excluded 33–48% inconsistently detected transcripts. Analysis of data from five time points over 48 h after infection identified 132 and 24 differentially expressed genes in epithelial cells and lymphocytes, respectively. Genes involved in protein synthesis, the cell cycle, structure and metabolism were affected. The magnitude of gene-expression changes ranged from 0.62 to 1.62 and early gene induction was followed by downregulation after 4 h. Transcriptional changes in CRFK cells were distinct from those in lymphocytes, except for heat-shock cognate protein 71, which was induced at multiple time points in both cell types. These findings indicate that FIV infection induces transcriptional changes of a modest magnitude in a wide range of genes, which is probably reflective of the relatively non-cytopathic nature of virus infection.

INTRODUCTION

*Feline immunodeficiency virus* (FIV) is a lentivirus that induces an acquired immunodeficiency syndrome in cats. Infection of cats with FIV, akin to infection of humans with human immunodeficiency virus (HIV), results in progressive decline in the number and function of CD4+ T cells and concomitant immune dysfunction (Bendinelli et al., 1995; Burkhard & Dean, 2003; Hazenberg et al., 2000; Stevenson, 2003). Numerous mechanisms, including direct cytopathic death of infected cells, lack of thymic regeneration, apoptosis and shortened lifespan due to inadequate cytokine support from other immune cells, have been proposed to contribute to this cell loss in HIV and FIV infection (Burkhard & Dean, 2003; McCune, 2001; Yamazaki et al., 2004). Changes in the expression of cytokine or cytokine-receptor genes, e.g. macrophage colony-stimulating factor (M-CSF), interleukin 10 (IL10), IL12 p40, IL2, IL4, IL6, IL1β, stromal-derived factor (SDF)-1, tumour necrosis factor alpha (TNF-α), gamma interferon (IFN-γ) and macrophage inflammatory protein 1 alpha (MIP-1α), resulting from infection of lymphoid cell lines with FIV, have been reported (Dean & Pedersen, 1998; Lerner et al., 1998; Linenberger & Deng, 1999). Furthermore, major histocompatibility complex (MHC) class II molecules and genes involved in apoptotic pathways were upregulated after infection of lymphoid cell lines (Lerner et al., 1998; Yamazaki et al., 2004). These findings suggest that altered cytokine production and increased apoptotic cell death probably contribute to immune dysfunction in FIV infection. However, numerous additional cellular functions, such as protein synthesis, molecule trafficking and metabolism, are altered by lentivirus infection (Corbell et al., 2001; Geiss et al., 2000; van’t Wout et al., 2003) and are essential for cell function, but have not been characterized in FIV infection.
Like HIV, FIV is a lentivirus with tropism for lymphocytes and cells of monocytic origin. Immunodeficiency due to loss of CD4+ T cells evolves only gradually in infected cats, whereas neurological disease and malignant lymphoid tumours are more prevalent clinical manifestations. It has been suggested that the immunodeficiency of lentivirus infection is an epiphenomenon and that macrophage tropism and dysfunction, the hallmarks of ungulate lentivirus infection, may represent the true pathogenic manifestation of these virus infections (Weiss, 2003). As it is difficult to model slowly evolving complex cellular interactions in vivo, FIV infection is commonly investigated by using epithelial Crandell–Reese Feline Kidney (CRFK) or lymphoid cell lines. However, the restriction of in vivo FIV infection to cells of haematopoietic origin and the failure of most primary isolates to infect CRFK cells productively have called into question the biological relevance of CRFK cells as targets for FIV infection (de Parseval et al., 2004). Productive infection of CRFK cells by FIV corresponds to the presence of specific amino acid substitutions within the viral envelope (Vahlenkamp et al., 1997), occurred despite a premature truncation in orf-A (Phillips et al., 1990) and has been described only for molecular clones derived from subtype A viruses (Dean et al., 1999; Sparger et al., 1994). Although the sequential molecular events of virus entry have not been described for FIV, available data support a model whereby CD134, expressed predominantly on T cells, is the primary binding receptor for FIV and cell entry is mediated by interaction of the viral envelope gp95 with the ubiquitous chemokine receptor CXCR4 (Shimojima et al., 2004). Heparan sulfate proteoglycans and the adhesion molecule DC-SIGN may modulate attachment, but their exact role in vivo is currently unclear (de Parseval et al., 2004). While the interaction of different receptors and attachment factors with the viral envelope is increasingly understood, the biological response to FIV infection probably differs greatly between haematopoietic and epithelial cells, but is as yet poorly characterized.

The recent development of microarray technology has revolutionized the study of gene expression (Deyholos & Galbraith, 2001; Geiss et al., 2001). DNA microarrays allow simultaneous expression analysis of thousands of genes from a given source under specified conditions and offer the opportunity to study virus–host relationships at the molecular level (Bigger et al., 2001; Chang & Laimins, 2000; Kellam, 2001). The FIV–cat model is well characterized clinically and immunologically, but greater understanding of the host response to infection may allow better use of the model for experimental therapeutic and prophylactic interventions. Feline microarrays are not available, but feline genes have generally > 85% sequence similarity to their human counterparts (Leutenegger et al., 1998; Nishimura et al., 1998; Rimstad et al., 1995; Ritchey et al., 2001; Schijns et al., 1997), suggesting the valid use of human microarrays for transcriptional analysis. Therefore, the objectives of this study were: (i) to validate feline to human DNA cross-hybridization on microarrays as an investigatory tool; and (ii) to determine whether transcriptional changes induced by a well-characterized cloned virus in an epithelial cell line differ from those induced by a biological FIV isolate in primary feline lymphocytes.

**METHODS**

**Cell culture.** CRFK cells (ATCC) were maintained in minimum essential medium (MEM) with 10% FBS, 2 mM l-glutamine, 5 mM HEPES, 0.1 mg penicillin–streptomycin (Life Technologies) ml−1 at 37°C and 5% CO2. CRFK cells are adherent epithelial cells and were passaged as necessary with 0.05% trypsin. Blood (40–50 ml) was collected by venipuncture from healthy, FIV-negative adult cats into 10 ml Vacutainer tubes containing sodium heparin (BD Biosciences) and mononuclear cells were isolated by Ficoll-Hypaque (Life Technologies) density-gradient centrifugation. The mononuclear cells were seeded at 10⁶ cells (ml RPMI 1640 medium)−1 (10% FBS, 2 mM l-glutamine, 5 mM HEPES, 0.1 mg penicillin–streptomycin ml−1) in 25 cm² flasks and stimulated with concanavalin A (ConA, 10 mg ml⁻1; Sigma) and human recombinant IL2 (hrIL2, 100 U ml⁻1; NIH AIDS Research and Reference Reagent Program).

**Viruses and infections.** FIV 34TF10 is a cloned isolate of FIV subtype A prepared through passage in CRFK cells and was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, MD, USA, from J. Elder. UsgaB01 is a biological isolate of FIV subtype B (Reggett & Bienzle, 2004) and was prepared through two passages in feline lymphocytes.

CRFK cells were grown to 90–95% confluence and treated with polybrene (2-0 mg ml⁻¹; Sigma) for 1 h at 37°C. Cells were washed once with warm PBS and 19 TCID50 FIV 34TF10 in medium, or an equal volume of PBS, was added. Virus- and mock-infected cells were incubated for 2 h at 37°C to allow for virus adsorption and infection. Cells were then washed and resuspended in fresh medium and aliquots were cultured for 1, 2, 4, 24 and 48 h in 150 cm² flasks. Additional control experiments consisted of cells exposed to ‘spent’ culture medium from uninfected cells.

Lymphocytes were cultured for 3–5 days with hrIL2 and Con-A, counted and prepared at a density of 4×10⁶ cells (ml medium)−1. Approximately 10⁶ cells were seeded into eight separate 50 ml centrifuge tubes and cells were treated with polybrene for 1 h at 37°C. Cells were washed and infected with 16 TCID50 UsgaB01 for 2 h or mock-infected with ‘spent’ medium. Cells were washed and resuspended in 10 ml fresh medium and aliquots were cultured for 1, 2, 4 and 24 h in 25 cm² flasks. Total RNA was isolated at each time point. Individual aliquots of CRFK cells and lymphocytes were infected on three separate occasions.

**p24 detection in supernatant of infected cells.** Supernatant samples were collected from FIV-infected cells at 48 h post-infection (p.i.) and examined for the presence of viral p24 protein by ELISA (PetCheck FIV antigen ELISA; Idexx) according to the manufacturer’s instructions.

**Cellular p24 detection.** Viral protein expression in FIV-infected lymphocytes was determined by flow-cytometric and light-microscopic detection of p24. Antibodies used were anti-FIV p24 (vpg50) and anti-FIV gp120 (vpg68; Serotec). For flow cytometry, lymphocytes were washed once with flow buffer (PBS with 5% bovine serum and 0.1% sodium azide) and fixed with Leukoperm A (Serotec), and 20 μl antibody was added for 30 min at room temperature in the presence of detergent (Leukoperm B). Cells were washed again with flow buffer and incubated with a secondary anti-mouse IgG antibody linked to fluorescein isothiocyanate (Serotec) in the presence of detergent. After a final wash, fluorescence was...
detected immediately in a FACScan (BD Biosciences) cytometer and results were analysed with CellQuest software. In control samples, the primary antibody was omitted or an irrelevant isotype-matched antibody was added. For immunocytochemistry, lymphocytes or CRFK cells were centrifuged directly onto slides (Cyto centrifuge; Thermo-Shandon). The slides were fixed in acetone for 5 min and primary antibodies were added undiluted for 30 min at room temperature. Slides were washed with Tris-buffered saline (TBS), secondary antibodies conjugated to a polymer with linked enzyme (EnVision; DAKO Cytomation) were added and specific colour was developed with a chromogenic enzyme substrate. Control slides were prepared in each case by omission of the primary antibody.

RNA isolation. Total RNA was isolated from mock- and HIV-infected cells at 1, 2, 4, 24 and 48 h.p.i. by using a GenElute Mammalian Total RNA kit (Sigma) according to the manufacturer’s instructions. For control experiments, RNA was isolated from uninfected cells grown in culture for up to 7 days and from cells treated with ‘spent’ medium. RNA was eluted into 75 µL RNase-free water and trace genomic DNA was removed by treatment with 7·5 U DNase I (Sigma). DNase was inactivated with 50 mM EDTA, RNA quality was assessed by capillary electrophoresis (BioAnalyser 2100; Agilent Technologies) and the samples were stored at −84 °C.

Microarray hybridization. 1-7 K cDNA microarrays (Ontario Cancer Institute, Toronto, ON, Canada), composed of approximately 1700 human expressed sequence tags, each spotted in duplicate, were used in this study. Details of the individual genes and the format of the array are available at www.microarrays.ca/. Approximately 10 µg total RNA was reverse-transcribed in 50 µL 50 mM Tris/HCl (pH 8·3), 75 mM KCl, 3·0 mM MgCl2, 3·8 µM anchor T primer, 0·5 mM dATP, dCTP and dGTP, 0·15 mM dITTP, 0·15 mM aminothiol-dUTP, 10 mM DTT and RNase-free water. The samples were incubated at 65 and 42 °C for 5 min each and 500 U SuperScript II RNase H− reverse transcriptase (Invitrogen) was added to each sample for 2 h at 42 °C. Reverse transcriptase was inactivated at 95 °C for 5 min and the mixture was cooled rapidly to stop the reactions. To hydrolyse any remaining RNA, 10 µL 1 M NaOH was added and samples were incubated at 65 °C for 5 min. Reactions were neutralized with 10 µL 1 M HCl and 5 µL 1 M Tris/HCl (pH 7·5). The final cDNA was centrifuged through PCR purification columns (Millipore). cDNA samples were labelled with Alexa 555 and 647 dyes (Molecular Probes) by incubation at room temperature in the dark for 1 h. Unincorporated dye was removed (QIAQuick PCR purification kit; Qiagen) and the cDNA was precipitated with 3 M sodium acetate, glycogen and 1 vol. 100 % ice-cold 2-propanol. Pellets were washed with 2 vol. 70 % ethanol and resuspended in 2–5 µL RNase-free water.

The hybridization buffer consisted of 80 µL DIG EasyHyb (Roche) and 4·0 µL calf thymus DNA and was heated to 65 °C for 2 min. Both cDNA samples were added to 30 µL hybridization buffer and incubated at 65 °C for 3 min. This mixture was pipetted onto the DNA microarray chip and covered with a 24 × 30 mm glass coverslip. The chip was then incubated at 37 °C for 18 h. Each microarray experiment was repeated with reciprocally labelled cDNA to reduce variation from dye bias. Therefore, two arrays were prepared for each of three replicates at each time point.

Signal detection and analysis. After incubation, the arrays were washed three times in separate containers of 1 × sodium chloride/ sodium citrate (SSC), 0·1 % SDS at 50 °C for 15 min. Remaining detergent and salt were removed by washes in 1 × SSC and 0·1 × SSC and the chip was dried by centrifugation at 500 r.p.m. for 5 min. Microarray slides were scanned with a GenePix scanner 4000A (Axon Instruments). Images were acquired and preliminary analysis was performed with GenePix Pro 3.0 software (Axon Instruments). During image acquisition, the photomultiplier tube gains for both 635 nm and 532 nm lasers were adjusted to yield dye intensity between 0·95 and 1·05.

Microarray data analysis. During scanning and image acquisition, spots were filtered if six or fewer pixels were greater than the background intensity, if the border of one spot overlapped that of another, if the spot had an irregular shape or position, if the colour or intensity of one spot differed from that of its duplicate or if the local background was of an intensity that would interfere with an accurate assessment of a spot’s expression value. After filtering, data were print tip-normalized to all unflagged gene spots without background subtraction by using r-gui software (www.r-project.org). Across-slide normalization was applied to all slides from a specific time point or from a specific set of control experiments. After normalization, data were filtered again with Gene Traffic software (lobion Informatics), which flagged spots automatically if their intensity was less than the local or mean entire background, or less than 100.

Statistically defined expression changes in all experiments were identified with the microarray analysis program SAM (Significance Analysis of Microarrays, Department of Statistics, Stanford University, Stanford, CA, USA). A range of SAM delta values was assessed to identify different levels of gene changes in proportion to false-discovery rates (FDRs). Details of the SAM program have been reported previously (Cui & Churchill, 2003; Reiner et al., 2003; Tusher et al., 2001).

To identify non-specific or inconsistent hybridizations, four arrays were hybridized to cDNA from different populations of uninfected cells and two arrays were hybridized to cells exposed to ‘spent’ culture medium. Data from uninfected cells were analysed individually and in combination with data from medium-treated cells, as described above. Genes expressed differentially in control experiments, or eliminated by filters in three or more of six replicate infection arrays, were excluded before analysis with SAM. Lymphocyte microarray data were subjected to SAM before filtering with Gene Traffic. FDR calculations for all microarray experiments included genes that were identified by SAM twice as a result of duplicate spotting, as well as those that were eliminated due to differential expression in uninfected or medium-treated CRFK cells.

Real-time PCR quantification. RNA was isolated and reverse-transcribed into cDNA as described above. For five genes expressed differentially across multiple array experiments and at different levels, primers were designed to amplify 100–200 bp, encompassing a region conserved between the human gene sequence present on the microarray and the corresponding mouse gene sequence. This strategy allowed for the amplification of feline genes that lacked published sequence data and yielded predicted amplicons of 177, 200, 155 and 160 bp for feline genes encoding heat-shock cognate protein 71 (HSC-71), Yes-associated protein-65 (YAP-65), insulin-like growth factor-binding protein-2 (IGFBP-2) and small nuclear ribonucleoprotein polypeptide-E (SnRNP-E), respectively (Table 1). Additional primers amplified 190 bp of the housekeeping gene feline β-actin. For each primer set, standard curves were generated to quantify gene-expression levels relative to β-actin. Standard curves were accepted if the slope was between −3·2 and −3·4, which corresponded to a reaction efficiency of approximately 2·0. Specific coefficient files were generated from the standard curve for each gene (LightCycler Relative Quantification Software, version 1.0; Roche). In each reaction, 2 µl purified cDNA from uninfected and infected cells was amplified with 2–3 mM MgCl2, 0·5 µM each primer and 2·0 µL LightCycler-FastStart DNA Master SYBR green I reaction mix (Roche). Samples were denatured for 10 min at 95 °C followed by 40 cycles of 95 °C for 5 s, 55 °C for 5 s and 72 °C for 8 s, with a final extension at 72 °C for 30 s, and melting of the amplicons to 95 °C at rate of 0·1 °C s−1. Finally, the reactions were
cooled at 40 °C for 30 s. PCRs were repeated three times for each gene and all novel amplicons were sequenced. Lack of primer-dimer formation and generation of single amplicons was confirmed by melting-curve analysis and gel electrophoresis.

**Sample preparation for sequencing.** Amplicons were purified (QIAquick PCR Purification kit; Qiagen) and concentrations were estimated by comparison to a low DNA mass ladder (Invitrogen) in agarose gels. DNA concentration was adjusted to 30 ng µl⁻¹ and sequences were determined by the BigDye Terminator method on an ABI Prism 377 DNA Sequencer (Molecular Supercentre, University of Guelph, Guelph, ON, Canada). Sequencing primers were the same as those used for PCR amplification.

**Western blotting of HSC-71.** Aliquots of FIV-infected and uninfected CRFK cells (10⁷ cells at 1 h p.i.) were lysed in 0.5 % Triton X-100 buffer containing 10 µg leupeptin ml⁻¹ and 1 mM PMSF. Control (20 µg HeLa cell lysate; Stressgen) and experimental cell lysates were separated in reducing polyacrylamide gels and transferred to PVDF membranes. The blots were blocked with 5 % BSA in Tris/PBS overnight at 4 °C and incubated for 1 h with a 1:2000 dilution of rabbit anti-HSC-71 polyclonal antibody (Stressgen). After three washes with PBS containing 0.05 % Tween 20, the blots were incubated for 1 h with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody (DAKO Cytomation) and bound antibodies were detected after exposure to diaminobenzidine chromogen.

### RESULTS

#### Efficiency of virus infection

Cytopathic effects are uncommon with FIV infection of lymphocytes or epithelial cells. Therefore, the proportion of virus-infected lymphocytes was estimated by flow-cytometric and immunocytochemical detection of viral protein expression. Antibodies directed against the viral envelope protein gp120 identified fewer than 3 % infected cells (data not shown), whereas intracellular FIV p24 expression was detected consistently in 30–36 % of cells at 24 h p.i. (Fig. 1). Infection of CRFK cells was assessed at 24 h p.i. by immunocytochemistry and at 48 h p.i. by detection of FIV p24 antigen in culture supernatants using ELISA. At 24 h p.i., 48–61 % CRFK cells contained detectable intracellular p24 antigen and at 48 h p.i., the p24 concentration in supernatants was 2–40 µg ml⁻¹ (data not shown).

#### Cross-species hybridizations

Initially, feline cDNA from uninfected CRFK cells was hybridized to human DNA to determine hybridization

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**Table 1. Real-time PCR primers for feline genes (5’→3’)**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-Actin</td>
<td>TGTCTGCCTGAGGCTTGCGT</td>
<td>GTGCTGTAAGATCTGTAGC</td>
</tr>
<tr>
<td>Heat-shock cognate protein 71</td>
<td>CTCCCTTTTCCCTTGGTATT</td>
<td>CAGGTGTATGCTGTTGCTC</td>
</tr>
<tr>
<td>Insulin-like growth factor-binding protein-2</td>
<td>CCGAATCTGTGCAAAGCAT</td>
<td>TAGAAGAGATGACACTCGGG</td>
</tr>
<tr>
<td>Yes-associated protein-65</td>
<td>GATGATTCGAGCTCATTG</td>
<td>ATCCTGGACAGAAGATGTC</td>
</tr>
<tr>
<td>Small nuclear ribonucleoprotein-E</td>
<td>ATGAGCAAGTAATATGCC</td>
<td>GACCCAGTTGTWTCTGTG</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Immunochemical detection of FIV p24 in lymphocytes at 2 (a) and 24 (b and c) h p.i. Cells were infected with 16 TCID₅₀ USgaB01 or mock-infected. They were then fixed and stained with anti-FIV p24 or control antibodies. Shaded areas represent background fluorescence; FIV p24-specific fluorescence was detected in 1.8 % (a) and 34.2 % (b) of cells. Intracellular p24 was detected by immunocytochemistry in lymphocytes (c). Bar, 10 µm.
efficiency and to characterize transcriptional variability in feline cells during culture. After data were filtered and normalized, between 600 and 700 genes were found to be suitable for further analysis. Of these, 66 genes (see Supplementary Table S1, available in JGV Online) were identified as being expressed differentially in separate aliquots of cultured CRFK cells or after exposure to ‘spent’ culture medium. SAM delta values of 0 ≤ 28 were chosen, corresponding to an FDR of 42 % (Fig. 2; Table 2).

CRFK cells

Data filtration and normalization criteria selected 900–1000 genes for analysis at each of five time points p.i. Of these, 132 genes were identified as being significantly differentially expressed. Delta values were set to identify a reasonable number of differentially expressed genes with a mean FDR of 39 % (Table 2). Most expression changes occurred immediately after infection, with 96 % (127 of 132) of genes expressed differentially within the first 4 h p.i. (see Supplementary Tables S2–S5, available in JGV Online) and upregulation was more common than downregulation (63 vs 37 %). At 1 h p.i., 52 genes were transcriptionally changed and 46, 29, three and two genes were changed at 2, 4, 24 and 48 h p.i., respectively. The ratio for each gene represents the mean change in expression of non-filtered genes for all replicate experiments, with the SD for each mean ratio indicated (see Supplementary Tables, available in JGV Online). The clone ID corresponds to the CloneID in the SOURCE gene report database available from Stanford University at www.genome-ww5.Stanford.edu. All microarray results with annotations have been deposited at the European Molecular Biology Organization Bioinformatics Institute, Array Express Database, files E-MEXP-203, E-MEXP-204 and E-MEXP-205.

Lymphocytes

In FIV-infected lymphocytes, approximately 825 genes were suitable for analysis (Table 2). Twenty-four genes were identified as being significantly differentially expressed, with a mean FDR of 46 % (Table 2). As in FIV-infected CRFK cells, most differential expression was observed early after infection and 71 % of genes were upregulated. At 1, 2, 4 and 24 h p.i., there were eight, three, ten and three transcriptionally altered genes, respectively (see Supplementary Tables S6–S9, available in JGV Online). Reduced viability of primary lymphocytes with RNA degradation precluded efficient hybridization at 48 h p.i. (data not shown).

Biological function of differentially expressed genes

Genes with differential expression in infected CRFK cells and lymphocytes were categorized based on potential cellular function (see Supplementary Tables S2–S9, available in JGV Online). Although several gene products were involved in multiple cellular activities, each gene was classified into only one functional group. The products of the majority of differentially expressed genes with known function were involved in protein synthesis, cell metabolism or were expressed on the cell surface. Genes involved in transcriptional regulation, cell structure, intracellular signalling, cell-cycle regulation, host response to pathogens, DNA replication and RNA splicing were also identified.

Table 2. Summary of statistical parameters of gene-expression changes in FIV-infected and uninfected cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. genes analysed</th>
<th>Mean delta value</th>
<th>No. differentially expressed genes</th>
<th>Mean FDR* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected and medium-treated CRFK cells</td>
<td>675</td>
<td>0.228</td>
<td>66</td>
<td>42</td>
</tr>
<tr>
<td>Infected CRFK cells</td>
<td>950</td>
<td>0.146</td>
<td>132</td>
<td>39</td>
</tr>
<tr>
<td>Infected lymphocytes</td>
<td>825</td>
<td>0.164</td>
<td>24</td>
<td>46</td>
</tr>
</tbody>
</table>

*False-discovery rate (FDR) calculation includes genes that were identified twice due to duplicate spotting or eliminated due to differential expression in control experiments.
Confirmation of microarray results

Although SAM is a widely accepted statistical method for the detection of differentially expressed genes by microarray analysis (Reiner et al., 2003), transcriptional changes of five different genes were confirmed with quantitative PCR. Expression relative to β-actin was determined for three genes from the CRFK (HSC-71, YAP-65, IGFBP-2) and two genes from the lymphocyte (HSC-71, SnRNP-E) datasets. Agarose-gel electrophoresis and melting-curve analysis of PCR products confirmed the presence of a single band of the correct size and the lack of primer-dimer formation in each case, respectively (data not shown). Amplification by PCR supported microarray expression data and, for all but one gene (SnRNP-E), the magnitude of the change was much greater by quantitative PCR than by microarray analysis (Table 3). The sequences of feline HSC-71, IGFBP-2, YAP-65 and SnRNP-E amplicons were 93, 99, 87 and 96 % identical to the corresponding human gene sequences, respectively.

Protein expression

The presence of the protein product of a gene, HSC-71, upregulated at multiple time points after FIV infection in CRFK cells and lymphocytes, was assessed by immunoblotting. The protein was detected in uninfected and infected CRFK cells and relative expression was greater in infected cells (Fig. 3).

DISCUSSION

The first large-scale analysis of gene-expression changes from FIV infection by using microarrays is reported here. Microarrays are a powerful tool for unbiased analysis of the transcriptome through concurrent assessment of 103–105 genes under defined and objective conditions. Refinements toward smaller quantities of hybridizing nucleic acids and more specific oligonucleotide origins permit increasingly tissue- and cell-specific applications (Higgins et al., 2004), which may eventually help to elucidate the complex but precise biology of cellular gene regulation in defined cells and multiple species.

**Table 3. Microarray and quantitative PCR gene-expression ratios**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Microarray</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRFK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-shock cognate protein 71</td>
<td>1·2</td>
<td>3·5</td>
</tr>
<tr>
<td>Insulin-like growth factor-binding protein-2</td>
<td>0·92</td>
<td>0·41</td>
</tr>
<tr>
<td>Yes-associated protein-65</td>
<td>1·6</td>
<td>3·0</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-shock cognate protein 71</td>
<td>1·2</td>
<td>1·7</td>
</tr>
<tr>
<td>Small nuclear ribonucleoprotein polypeptide-E</td>
<td>1·1</td>
<td>1·4</td>
</tr>
</tbody>
</table>

**Fig. 3.** HSC-71 protein is increased in FIV-infected CRFK cells. Cell lysates of HeLa cells (lane 1), FIV-infected CRFK cells (lane 2) and uninfected CRFK cells (lanes 3 and 4) were separated by electrophoresis and transferred to membranes. Lanes 1–3 were probed with an antibody against HSC-71 and bound antibody was detected in all lanes with enzyme-labelled secondary antibody and chromogenic substrate. Relative densities are 1·00 (lane 1), 3·41 (lane 2), 0·61 (lane 3) and 0·00 (lane 4).

Within the context of FIV infection, we were interested in gaining a broader understanding of the effects of the virus on cell signalling, cell-cycle regulation, and induction of specific inflammatory and immune-response genes. A widely used epithelial cell line that supports replication of tissue culture-adapted FIV subtype A variants was investigated, as well as primary lymphocytes infected with a biological isolate of FIV. Human microarrays were chosen for this study, as the sequence of known feline genes is greater than 85 % identical to the corresponding human gene, particularly for genes of the immune system (Leutenegger et al., 1998; Nishimura et al., 1998; Rimstad et al., 1995; Ritchey et al., 2001; Schijns et al., 1997). Arrays specific for cats are not yet available, but cross-hybridization to human nucleic acids may be sufficient under carefully adjusted stringency conditions and with robust statistical analysis to identify specific changes. From six experiments to address homeostatic gene hybridization, 66 genes were identified by statistical analysis as being expressed differentially and between 600 and 700 genes passed the applied filter criteria. The 66 genes with variable signal detection represented 3·48 % of all genes assessed and were probably excluded due to intermittent expression during in vitro culture, the presence of low numbers of transcripts that hybridized erratically or weakly, or variation inherent in RNA isolation, cDNA labelling, array hybridization or scanning procedures. This proportion of filtered genes was comparable to results from same-species hybridizations (Chang & Laimins, 2000; Chismar et al., 2002; Corbeil et al., 2001; van’t Wout et al., 2003) and it is concluded that feline to human cDNA hybridization on microarrays yielded acceptable results for experimental investigation. However, these experiments emphasized the importance of replicate hybridizations to clearly delineate variability inherent
in the procedure. Similar levels of background expression variability have been observed when porcine and non-human primate DNA was hybridized to human oligonucleotides (Chismar et al., 2002; Moody et al., 2002). In these studies, 6 and 8% of genes were eliminated due to variable detection, respectively, but a clear correlation between sequence similarity and variable signal detection was not identified. In addition, pig to human hybridization yielded similar signal detection rates as human to human hybridization. Cats are genetically more similar to humans than pigs (Thomas et al., 2003), suggesting that inherent variability in gene expression or technique, rather than limited sequence similarity, determined the consistency of hybridization signals.

Analysis of gene-expression changes in the different cell types identified 132 differentially expressed genes in CRFK cells and 24 genes in lymphocytes. These genes represented 13 and 3% of the total genes analysed in CRFK cells and lymphocytes, respectively. In both cell types, the majority of differentially expressed genes were upregulated and most transcriptional variation occurred within the first 4 h p.i. Predominant upregulation of transcription was identified by microarray analysis in most virus infections and has been related to induction of cellular defence mechanisms (Bigger et al., 2001; Chang & Laimins, 2000; Corbeil et al., 2001; Cuadras et al., 2002; Geiss et al., 2000; Guerra et al., 2003; Jones & Arvin, 2003; Mossman et al., 2001; Otsuka et al., 2003; Pietiäinen et al., 2000). The cumulative effect on the cell-cycle genes induced by FIV infection of CRFK at 1 h p.i. promoted transition from G1 to S phase. Chromosomal changes during DNA synthesis prior to cell division may facilitate the integration of double-stranded proviral DNA (Mitchell et al., 2004). This effect on cell-cycle genes was only observed at 1 h p.i. and was restricted to CRFK cells, which may indicate a unique virus–cell interaction whereby FIV 34TF induces early DNA synthesis, resulting in cell proliferation and enhanced virus integration relative to infection of lymphocytes. Downregulation of cell-cycle genes was prominent and attributed to a G2 arrest of viral protein R (Vpr) in HIV infection (Iordanskiy et al., 2004). A similar effect on the cell cycle was not observed during later time points in this investigation.

The majority of induced protein-synthetic genes at 1 h p.i. belonged to the large family of ribosomal proteins, which may indicate broadly enhanced protein synthesis. Cellular protein synthesis early after virus infection may be polarized toward HIV or herpesviral protein production (Corbeil et al., 2001; Mossman et al., 2001) and may occur in FIV infection. Of particular interest was induction of the gene for ribosomal protein L37a, which encodes heparin-binding peptides (Rohde et al., 1996). The presence of heparan sulfate proteoglycans (HSP) on the surface of CRFK cells enhanced CXCR4-mediated infection by FIV 34TF in the absence of CD134 (de Parseval et al., 2004), suggesting that induction of this gene may promote further FIV binding.

Members of the chaperonin family of proteins were induced at numerous time points and in CRFK and lymphoid cells. HSC-71 (also known as HSC70 and HSPAB), like other chaperonins, associates transiently with polypeptides to facilitate their correct folding during translocation through mitochondrial and endoplasmic membranes (Bhattacharyya et al., 1995; Robert, 2003). HSC-71 has several reported functions of interest in retrovirus infections: overexpression of HSC-71 suppressed the cell-cycle arrest induced by Vpr of HIV and thereby reduced apoptosis of infected cells (Iordanskiy et al., 2004). Furthermore, surface-expressed HSC-71 interacted with envelope gp46 of the human T-cell lymphotropic virus (HTLV) and was proposed to mediate syncytium formation and therefore cell-to-cell virus transmission (Sagara et al., 1998). In addition, HSC-71 was shown to be part of a CD14-independent lipopolysaccharide signal-transmitting cluster on monocytes containing other heat-shock proteins, growth differentiation factor-5 and CXCR4 (Triantafilou et al., 2001), and to mediate tumour-antigen recognition in three-dimensional cell aggregates (Dangles-Marie et al., 2003). All of these functions of HSC-71 are potentially relevant to FIV infection.

SnRNP-E, which is involved in splicing of pre-mRNA molecules and possibly in splicing of viral mRNA transcripts, was induced in lymphocytes and may aid generation of functional FIV env gene transcripts (Tomonaga et al., 1993). The gene for IGFBP-2, downregulated in CRFK cells at 4 h p.i., is frequently altered by virus infections (Kang-Park et al., 2001; Mossman et al., 2001; Otsuka et al., 2003; Pietiäinen et al., 2000). IGFBP-2 has been proposed as the receptor for granzyme B (Motyka et al., 2000), which would suggest an important role in host defence mechanisms.

Uregulation of genes encoding caspases was not observed in these array studies, although probes for the genes were present on the chip and caspases are highly conserved between cats and humans (Yamazaki et al., 2004). In a study of in vitro apoptosis in FIV-infected T-cell lines, only bcl-2 mRNA was reported to be upregulated, and not bax, caspase 3 or bcl-xL (Yamazaki et al., 2004). In our experiments, the most profound change in gene expression was found with YAP-65 (also known as YAP-1) at 1 h p.i. in CRFK cells. YAP-65 is a widely expressed nuclear transcription factor that is translocated to the cytoplasm after serine phosphorylation by the AKT protein kinase (Basu et al., 2003). Experimentally, overexpression of YAP-65 increased apoptosis following DNA damage (Basu et al., 2003). However, as no other apoptosis-related genes were upregulated by FIV infection, the pro-apoptotic function of YAP-65 may have been suppressed by phosphorylation or it may have additional cellular functions unrelated to apoptosis. The very gradual development of CD4 lymphopenia in FIV-infected cats may indicate that biologically relevant apoptosis occurs only late during infection and not as a direct effect of virus infection.
The breadth of genes affected by FIV infection was remarkable and ranged from those encoding receptors involved in electrolyte transport across the cell membrane to those with functions in the cell cycle and in protein synthesis. However, the relative change in gene expression was modest, in the range 0·62–1·62, and was comparable between CRFK cells and lymphocytes. Changes of a similar magnitude were described in HIV infection, whereas rotavirus infection caused upregulation of some genes by a factor of 17 (Corbeil et al., 2001; Cuadras et al., 2002; Geiss et al., 2000; Rojas et al., 2003; van’t Wout et al., 2003). Differences in statistical analysis may account for some of the discrepancy, as SAM tends to underestimate relative gene-expression changes on microarrays (Cui & Churchill, 2002) compared to fold-change methods. However, profound differences in the biology of acutely cytopathic and non-cytopathic viruses are probably reflected by the magnitude of changes in gene expression.

There was little overlap in the genes whose expression was changed in CRFK cells and lymphocytes. As replicates of cell infections with the same virus yielded highly reproducible results with relatively low SD values, the difference in gene expression between CRFK-cell infection by FIV 34TF and primary lymphocyte infection by USg aB01 probably reflected different cell responses. These distinct cell responses may result from different receptor usage and therefore dissimilar early cell signals, from differences in the activity of transactivating proteins or from sequence disparity in the viral long terminal repeat (de Parseval et al., 2004; Thompson et al., 1994). Changes detected in lymphocytes were generally fewer, but of similar magnitude to those in CRFK cells. The use of a mixture of lymphocytes may have obscured the number and extent of gene-expression changes that might be expected if purified CD4 T cells were assessed. However, the predominant in vivo target population for FIV infection remains disputed. Infection studies indicated that, soon after FIV infection, predominantly CD4 T cells contained provirus, whereas during the chronic phase, the greatest proviral burden was in B lymphocytes (Dean et al., 1996). In addition, macrophages and dendritic cells can be infected by FIV (Dean et al., 1999; Rogers et al., 2002), but in vivo proviral burden over time in these cells remains undefined. The distribution of the primary receptor for FIV, CD134, on different haematopoietic cells and the degree of dependence of field isolates of FIV on the use of CD134 are unclear. In our experiments, the source of lymphocyte mRNA was from mononuclear cells stimulated with ConA in the presence of IL2. These culture conditions enrich for CD4 lymphocytes (unpublished observations) and, thus, gene-expression changes probably reflected predominantly the responses of this cell population. Nevertheless, the admixture of lesser proportions of other lymphocytes may have obscured some transcriptional changes.

Cytometric and immunocytochemical analysis showed expression of FIV p24 protein in 1·8 and 34·2 % of lymphocytes at 2 and 24 h p.i., respectively. Whilst expression of p24 occurs relatively late during lentivirus replication (Lama et al., 1999) and detection of reverse-transcriptase activity may indicate a greater degree of infection, these parameters show that a relatively large proportion of cells contained integrated and transcriptionally active virus. Assessment of CRFK cells indicated similarly productive infection.

Microarray analysis, in particular with stringent data filtration and normalization, typically underestimates gene-expression changes that have subsequently been determined to be much greater by using real-time PCR or other quantitative techniques (Higgins et al., 2004; Rajpal et al., 2003). Similarly, quantification of gene expression by real-time PCR surpassed microarray estimates for most genes in this study. The filter criteria applied during this data analysis were relatively stringent and excluded genes with inconsistent or poor levels of hybridization. This level of stringency was chosen to maximize gene hybridization and to yield clear signals during scanning. Therefore, a variable proportion (38–55 %) of genes passed filter criteria for further analysis. Such stringent filtration criteria typically reduce analysable genes to the extent illustrated in rotavirus infection, where only 48 % of genes were retained for analysis (Cuadras et al., 2002; Rojas et al., 2003), and in kidney-specific expression analysis, where 39 % of genes passed filtration criteria (Higgins et al., 2004).

The FDRs calculated by SAM ranged from 39 to 46 % and were selected to optimize gene discovery relative to potentially falsely identified genes. SAM calculations for all experiments included genes that were identified twice as a result of duplicate spotting, as well as those that were discounted from control experiments, which increased calculated FDRs. FDRs correlated with the magnitude of differential gene expression and were increased in models where relatively minor transcriptional changes occurred (Reiner et al., 2003). Thus, although FDRs in this study were high, they are probably reflective of more widespread but subtle expression changes. Other methods, such as fold change, have been used to identify transcriptional changes in microarray data (Chang & Laimins, 2000; Corbeil et al., 2001; Pietiäinen et al., 2000). However, the fold-change method proved unreliable, with FDRs as high as 84 % (Tusher et al., 2001).

In summary, gene-expression patterns in FIV-infected cells have been examined. Cross-hybridization to human nucleic acids with the application of stringent experimental conditions was sufficient to detect changes in feline gene expression. It has been demonstrated that 132 and 24 genes were expressed differentially in FIV-infected CRFK cells and primary lymphocytes, respectively. Relative expression changes overall were modest, probably reflecting the non-cytopathic nature of virus infection. Transcriptional changes in epithelial cells and lymphocytes were distinct; commonly induced genes were involved in chaperoning protein synthesis.
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