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A glucocorticoid response element in the LTR U3 region of Friend murine leukaemia virus variant FIS-2 enhances virus production *in vitro* and is a major determinant for sex differences in susceptibility to FIS-2 infection *in vivo*

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The nucleotide sequence of the Friend murine leukaemia virus variant FIS-2 LTR has high identity with the closely related Friend murine leukaemia virus (F-MuLV) LTR, except for the deletion of one direct repeat, a few point mutations and the generation of a glucocorticoid response element (GRE) in the U3 region. The GRE can mediate gene induction by glucocorticoids, mineral corticoids, progesterone and androgens, and it has been shown that incorporation of a GRE(s) within the LTR can increase the transcriptional activity of retroviral enhancers. We have previously reported an increased early virus replication in male mice compared with female mice when infected with a virus containing the FIS-2 LTR and have proposed that the GRE might contribute to this sex difference. In the present study, we introduced a single point mutation in the GRE and performed comparative studies in NIH 3T3 cells and in young adult male and female NMRI mice. We found that significantly more virus was produced from NIH 3T3 cells infected with wt FIS-2 than from cells infected with the FIS-2 GRE mutant and that this difference was further augmented by glucocorticoids. The glucocorticoid antagonist RU486 inhibited virus production in a dose-dependent manner. The wt FIS-2 disseminated significantly faster than the FIS-2 GRE mutant in both male and female mice. There was no significant difference in the dissemination rate between male and female mice infected with the FIS-2 GRE mutant. Hence, the GRE in the FIS-2 LTR is one determinant of the significant sex difference in susceptibility to FIS-2 infection.

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INTRODUCTION

Friend immunosuppressive virus (FIS-2) is a low-oncogenic, immunosuppressive variant of Friend murine leukaemia virus, F-MuLV (Faxvaag *et al.*, 1993; Dai *et al.*, 1994, 1998). We have previously reported that young adult NMRI male mice are more susceptible to FIS-2 infection than female mice (Bruland *et al.*, 2001). The level of virus in serum, bone marrow and spleen was initially higher in male mice. Male mice were also more susceptible to FIS-2-induced immunosuppression, and together these results indicated a more efficient virus replication and dissemination in male mice. Studies with recombinant viruses between FIS-2 and the prototype F-MuLV clone 57 revealed that the FIS-2 LTR was one major factor contributing to the observed sex difference in early virus replication.

The nucleotide sequence of the FIS-2 LTR shows high identity with that of the F-MuLV LTR, except for the deletion of one of the two direct repeats (DRs) in F-MuLV LTR and a few point mutations (Dai *et al.*, 1994). The deletion of the second copy of the DR in the FIS-2 LTR has been associated with the generation of a binding site for the glucocorticoid receptor (GR), 5′-AGAACAGATGG-3′, at the 3′ end of the remaining DR (Dai *et al.*, 1994). This glucocorticoid response element (GRE) contains a conserved hexanucleotide, 5′-AGAACA-3′, defined as the GRE core sequence (DeFranco & Yamamoto, 1986; Miksicek *et al.*, 1986). The core motif also has affinity for the progesterone receptor and androgen receptor (Nelson *et al.*, 1999, and references therein). A glucocorticoid-regulated transcription of provirus through the binding of a GR to the GRE has been described for both simple retroviruses (Beato *et al.*, 1989; Celander *et al.*, 1988; DeFranco & Yamamoto, 1986; Miksicek *et al.*, 1986) and complex retroviruses such as bovine leukaemia virus (Niermann & Buehring, 1997) and human immunodeficiency virus (HIV) (Kolesnitchenko & Snart, 1992; Mitra *et al.*, 1995; Russo *et al.*, 1999; Soudeyns *et al.*, 1993). The best-studied steroid-inducible retrovirus is

mouse mammary tumour virus (MMTV), in which the promoter is silent in the absence of hormones. The MMTV provirus is rapidly induced to high transcription levels by glucocorticoid hormones as well as progesterins and androgens (Beato *et al.*, 1989; Cato *et al.*, 1986; Darbre *et al.*, 1986; Otten *et al.*, 1988; Schüle *et al.*, 1988). We have previously proposed that the presence of the GRE in the FIS-2 LTR could contribute to the significant sex difference in early FIS-2 replication (Bruland *et al.*, 2001). The present study was undertaken to investigate this hypothesis further.

METHODS

Mutagenesis of the GRE in the FIS-2 LTR U3 region. Routine recombinant DNA procedures, such as restriction enzyme digestion, treatment with modifying enzyme T4 DNA ligase, purification of DNA fragments, plasmid preparation, nucleotide sequence analysis and plasmid transfection were performed as specified by the product manufacturers. A mutation from A to G in the 3′ end of the conserved hexanucleotide 5′-AGAACA-3′, characterizing the GR binding site (DeFranco & Yamamoto, 1986; Miksicek *et al.*, 1986), was performed in the FIS-2 LTR U3 region (Fig. 1). Plasmid pUC19 ligated with FIS-2 DNA at *Eco*RI sites had previously been constructed and used to transform competent *Escherichia coli* DHα cells (Dai *et al.*, 1994). The Qiagen Plasmid Midi kit was used for plasmid isolation and the mutation was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The forward and reverse primers were designed using the program Oligo 4.1 and synthesized by Eurogentec. Both primers were from nt 8014–8039 covering the GRE in the FIS-2 U3 region. The product containing the pUC19/FIS-2 GRE mutant was transfected into *E. coli* XL-1 Blue supercompetent cells. Single colonies were harvested and amplified, and plasmids were isolated using the Wizard *Plus* SV Miniprep DNA Purification System Protocol (Promega). To verify the mutation, the DNA was sequenced using the BigDye Terminator Cycle Sequencing kit run on an ABI PRISM 377XL DNA Sequencer (PE Applied Biosystems). The primers, with nucleotide positions ranging from 630 to 7649 at the end of the FIS-2 *env* gene, were designed using the program Oligo 4.1 and synthesized on an Applied Biosystems 381A synthesizer. A 510 bp long sequenced fragment containing the mutation was cut out of the pUC19/FIS-2 plasmid at the two unique restriction sites *Cla*I and *Asc*I and replaced with the analogous fragment of FIS-2 in the parental pUC19/FIS-2 plasmid. After digestion with the restriction enzyme, the samples were separated by electrophoresis and the fragments were purified from the agarose gel. The parental pUC19/FIS-2 lacking the 510 bp fragment was ligated with the 510 bp fragment containing the mutation. This pUC19/FIS-2 GRE mutant plasmid was transfected into *E. coli* DH5α competent cells. Single colonies were harvested and amplified, and plasmids were isolated (see above). The pUC19 plasmid was removed from the virus sequence using *Eco*RI. After digestion, the fragments were separated by electrophoresis and the FIS-2 GRE mutant fragment was purified from the agarose gel and ligated.

Cell line, antibodies and hormones. Mouse NIH 3T3 fibroblasts were maintained in DMEM supplemented with 2·5–10 % newborn calf serum (NCS), 2 mM L-glutamine and 0·05 mg gentamicin ml^{-1}. Hybridoma cell lines 34 and 48, producing monoclonal antibodies against *gag*-encoded MA proteins and gp70, respectively (Chesebro *et al.*, 1981), were maintained in RPMI 1640 medium with 50 μM 2-mercaptoethanol and 10 % foetal calf serum. Cell-free supernatant was used as a source of primary antibodies. The synthetic glucocorticoid agonist dexamethasone and the natural agonist hydrocortisone (cortisol) were diluted in dH_2O to 20 mM stock solutions, which were dispensed into small aliquots and stored at −20 °C until the start of each experiment. The glucocorticoid antagonist RU486 (Mifepristone) was diluted in 95 % ethanol

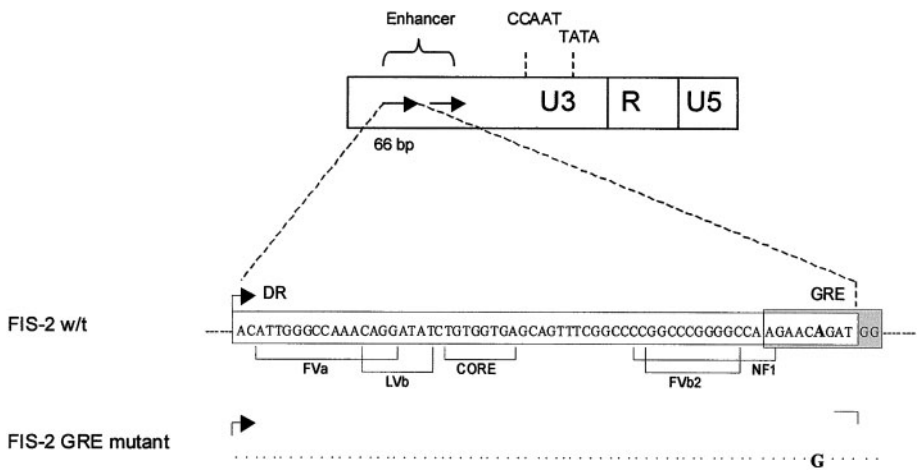

Fig. 1. Organization of a prototypical mammalian type C proviral LTR with its three regions U3, R and U5. The direct repeat (DR), the promoter (CCAAT) and the site of transcriptional initiation (TATA box) are located in the U3 region. The FIS-2 DR contains only one copy of the 66 bp sequence considered to be the viral enhancer (Golemis *et al.*, 1990). Binding sites for the identified viral core element (core) and nuclear factors (Speck & Baltimore, 1987; Manley *et al.*, 1989) are underlined in the FIS-2 DR sequence: Friend virus factor a and b2 (Fva, Fvb2), leukaemia virus factor b (LVb) and nuclear factor 1 (NF1). The grey box frames the GRE (5′-AGAACAGATGG-3′) in the FIS-2 LTR U3 region. The DR in the FIS-2 GRE mutant is homologous (indicated by dots) to wt FIS-2 except for the single point mutation from A to G at the end of the conserved hexanucleotide 5′-AGAACA-3′ (see Methods).

(50 mg ml^{-1}) and added to the cell culture medium. All control cultures contained the same concentration of ethanol. Hormones and the anti-hormone were purchased from Sigma.

Viruses. The viruses used in this study included a molecular clone of an immunosuppressive variant of Friend murine leukaemia virus, the N-tropic FIS-2 (Dai *et al.*, 1994) and the FIS-2 GRE mutant (see above). Virus stocks were prepared and titrated from supernatants collected from transfected cells as described previously (Dai *et al.*, 1998). The titres of the FIS-2 wt and FIS-2 GRE mutant stocks were $2{\cdot}2\times10^6$ and $2{\cdot}65\times10^6$ infectious units (IU) ml^{-1}, respectively. RT-PCR and subsequent sequencing verified the presence of the mutated GRE in the prepared virus stock. The reverse primer corresponded to a sequence in the LTR region (nt 8075–8095) and the forward primer corresponded to a sequence at the end of the *env* gene (nt 7630–7650), producing a fragment of 466 bp. Amplification was confirmed by electrophoresis and the RT-PCR product was purified (Qiagen PCR purification kit) and sequenced as described above. The stability of the single nucleotide exchange in the GRE core sequence after *in vitro* and *in vivo* replication was confirmed by RT-PCR and subsequent sequencing of the virus genome isolated from *in vitro*-infected NIH 3T3 cell culture supernatants and serum from infected mice, respectively.

RT assay. NIH 3T3 cells ($1{\cdot}5\times10^4$) were seeded into each well of 24-well plates. After attachment for 2–4 h, the medium was changed and the cells were incubated for 24 h with 5000 IU (0·3 IU per cell) of either wt FIS-2 or FIS-2 GRE mutant in fresh medium supplemented with polybrene and with different concentrations of dexamethasone or hydrocortisone and RU486. The medium was then replaced with a virus- and polybrene-free medium and the cells were further incubated with or without freshly added hormones and anti-hormone. RT activity of extracellular particles was used as a measure of virion production at different times post-infection (p.i.). RT activity was determined using a standard technique based on that of Goff *et al.* (1981). In brief, 800 µl cell-free culture supernatant was centrifuged at 15 000 ***g*** for 60 min and the pellet was resuspended in 25 µl dH_2O before 25 µl reaction cocktail was added. The reaction mixture was incubated at 37 °C for 1 h. Radioactivity was counted in a β-scintillation counter.

MTT assay. An experimental *in vitro* design similar to the one described above was performed with cells grown in 96-well plates. The cell number, virus concentration and dexamethasone concentration were adjusted to those of the 24-well plates and the two cell cultures were run in parallel. The growth of cells was assessed by the MTT reduction assay, essentially as described by Mosmann (1983).

Flow cytometric quantification of virus-infected NIH 3T3 cells. All steps were performed at 0–4 °C. Adherent NIH 3T3 cells were incubated in PBS containing 9 g EDTA l^{-1} for 2 min before being harvested by gentle scraping and low-speed centrifugation. A mix of hybridoma supernatants 34 and 48 (described above) was added. The cell suspensions were transferred to a 96-well plate where the cells were incubated for 2 h. After three washes in PBS containing 0·1 % BSA, the cells were further incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Dako). The cells were then washed three times, resuspended in PBS and analysed with a FACScan flow cytometer (Becton Dickinson). Five-thousand cells were analysed per sample on a single-cell basis and displayed as frequency distribution histograms. Dead cells and debris were gated out of analysis on the basis of a forward light scatter signal. The results were expressed as the percentage of cells that stained positive after subtraction of the stained cells in the control samples. The number of positive cells per well was calculated from the average number of cells in the corresponding wells.

Animals Seven-week-old male and female NMRI mice were purchased from the Bomholdt Gaard Breeding Research Center, Rye, Denmark. The mice were kept in groups of five under conditions of controlled temperature and a 12 : 12 h light/dark cycle, with food pellets and water *ad libitum*. The handling and treatment of the experimental animals were performed according to the regulations of the Norwegian Law on the Care and Use of Laboratory Animals.

Determination of virus titres in serum and spleen. All mice were bled from the cervical vein under anaesthesia (1 : 1 mixture of hypnorm : dormicum) for viraemia analysis and subsequently sacrificed by cervical dislocation. Viraemia titres were determined by end-point dilution (Grist *et al.*, 1990), as described previously (Bruland *et al.*, 2001). Preparation of single-cell suspensions of splenocytes and detection of virus-positive cells by indirect immunofluorescence were performed as described by Dai *et al.* (1998). The virus load in spleens was determined by end-point dilution of single-cell suspensions: 10-fold dilutions were transferred into four wells (1·5 cm^2) of subconfluent monolayers of NIH 3T3 cells of up to 10^7 spleen cells per well. After two rounds of trypsination and 1 : 10 dilution, the cells were prepared for immunofluorescent detection of positive cells. The number of productively infected nucleated splenocytes was calculated from the lowest dilution with three or more positive wells.

Determination of antibodies directed against viral membrane proteins on FIS-2-infected cells. Subconfluent monolayers of NIH 3T3 cells transfected by FIS-2 clones were grown in 96-well plates. A twofold dilution of sera from infected and uninfected (control) mice was used as a source of primary antibody. The cells were prepared for immunofluorescence as described by Dai *et al.* (1998). The antibody titres were given as the highest dilution giving immunofluorescent signals on the infected cells.

Statistical methods. Student's *t*-test was used to determine the level of significance between the means of samples in the *in vitro* experiments. Statistical analysis of viraemia titres and number of infected splenocytes were performed on $\log_{10}$-transformed data with the two-tailed Mann–Whitney U rank sum test for independent samples, using the exact test option (corrected for ties). A value of $P\leqslant0{\cdot}05$ was considered significant. The rank correlation between viraemia titres and the number of productively infected cells in the spleen at 8 days p.i. was analysed by calculating Spearman's rho. Correlation was significant at the 0·01 level (two-tailed). The SPSS version 10.07 software was utilized for all statistical analyses.

RESULTS

The presence of an intact GRE in the FIS-2 LTR U3 region enhances virus production *in vitro*

We first investigated whether the GRE in the FIS-2 LTR could enhance virus production from *in vitro*-infected cells. The fibroblast cell line NIH 3T3 was used because it is highly susceptible to Friend virus infection and has been demonstrated to have GRs (Höck *et al.*, 1989). When we compared the RT activity in the cell culture supernatants at 72 h p.i. with wt FIS-2 and the FIS-2 GRE mutant, we found that the presence of an intact GRE increased virus production significantly, both in cultures grown with dexamethasone and in cultures grown without glucocorticoid (Fig. 2).

To determine whether differences in cell numbers were indirectly causing an increased virus production from wt FIS-2-infected cell cultures, the cells in each well were

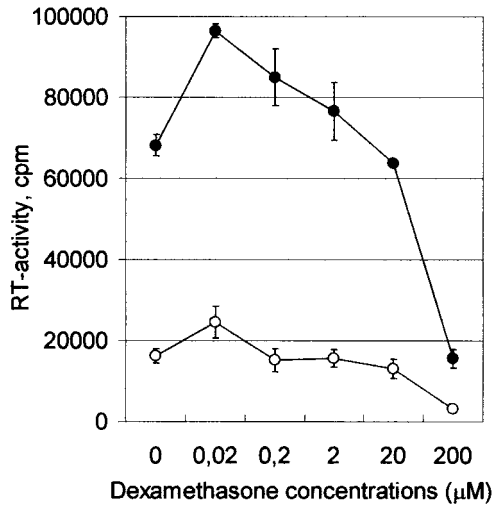

Fig. 2. Virus production from wt FIS-2 (●) and FIS-2 GRE mutant (○) *in vitro*-infected NIH 3T3 cells grown with 10% serum and at different concentrations of dexamethasone. Each point represents the average RT activity (c.p.m.) from three to six wells ± SD at 72 h p.i. The results are representative of four independent experiments.

harvested by trypsination and counted with a haemocytometer. There was no significant difference (*P* values 0·2–0·6) in the cell number between cultures infected with wt FIS-2 and the FIS-2 GRE mutant (FIG. 3A). The average number of uninfected and untreated NIH 3T3 cells was $1{\cdot}0 \pm 0{\cdot}3 \times 10^5$ cells per well ($n = 4$), which is in the normal range after 72 h p.i. (24 h average duplication time). Low doses of dexamethasone seemed to stimulate cell proliferation. This was reflected in the shapes of the dose-response curves (Fig. 2). Hormonal influence on cell viability was also assessed by the MTT reduction assay. At low concentrations of dexamethasone, no significant influence on cell viability was noticed in this assay. At high concentrations, dexamethasone down-regulated cell proliferation in a dose-dependent manner (Fig. 3B), which could explain the down-regulation of RT activity in cell cultures supplemented with high dexamethasone concentrations (Fig. 2).

In order to confirm that the enhanced RT activity in wt FIS-2-infected cultures was not the result of a relatively higher wt FIS-2 dose (IU) used to infect the cells, we compared the RT activity in different dilutions of wt FIS-2 and FIS-2 GRE mutant stocks. The dose-response curves for the wt FIS-2

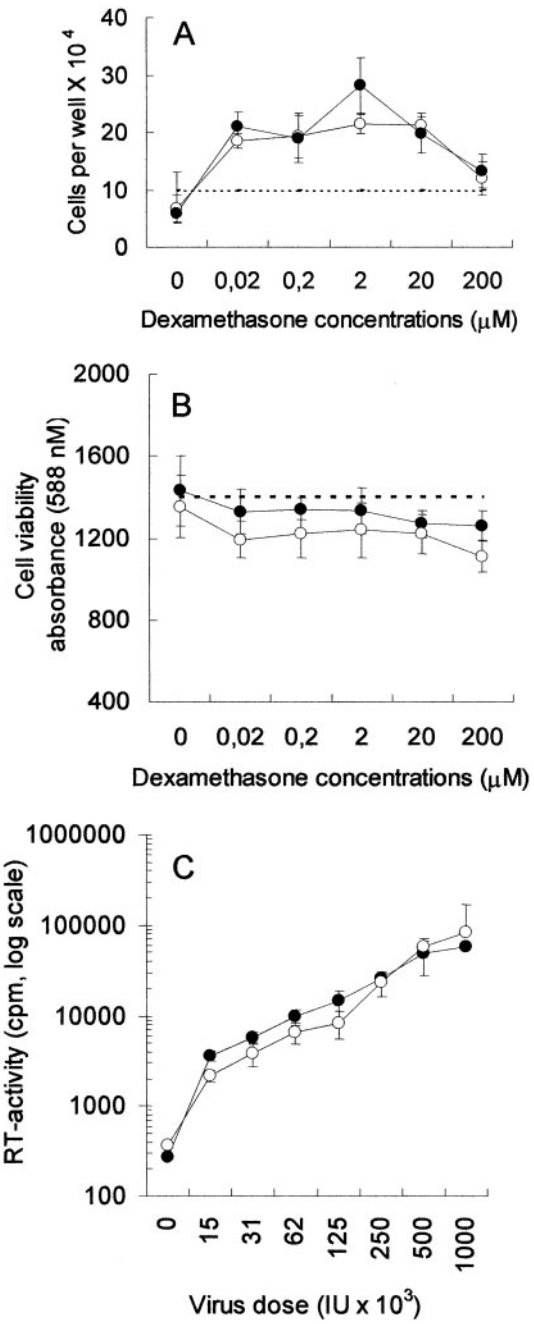

Fig. 3. Tests for cell number and virus dose. (A) Number of cells per well at 72 h p.i. Each point represents the average cell number in three wells infected with wt FIS-2 (●) or the FIS-2 GRE mutant (○). The bars show SD. The dotted line indicates average cell number from four wells of uninfected NIH 3T3 cells grown in the absence of dexamethasone ($1 \pm 0{\cdot}3 \times 10^5$). The results are representative of three independent analyses. (B) Cell viability was assessed using the MTT assay and expressed as absorbance (588 nm) per well. Each point represents average value ± SD from six (with dexamethasone) or 12 (without dexamethasone) cultures infected with wt FIS-2 (●) or the FIS-2 GRE mutant (○). The dashed line indicates the average absorbance in control cultures grown in the absence of both virus and hormones ($n = 12$ wells). Similar data were obtained in two independent experiments. (C) Control of virus dose. Different dilutions of wt FIS-2 (●) and FIS-2 GRE mutant (○) virus stocks were analysed for RT activity. Each point represents average c.p.m. ± SD from three parallel experiments and the result are representative of three independent analyses.

and the FIS-2 GRE mutant were similar (Fig. 3C). Thus, the higher RT activity in wt FIS-2-infected cultures compared with FIS-2 GR mutant-infected cultures was neither due to unequal cell numbers in the wells nor due to the use of a higher wt FIS-2 inoculation. The single point mutation was also stable, as verified by RT-PCR and subsequent sequencing of the virus genome isolated from *in vitro*-infected cultures (see Methods).

More virus is produced from wt FIS-2-infected cells than from FIS-2 GRE mutant-infected cells, and wt FIS-2 production can be further augmented by dexamethasone

Subsequently, we examined whether more virus was produced from FIS-2-infected cells compared with FIS-2 GRE mutant-infected cells and whether wt FIS-2 production could be augmented by glucocorticoid. Based on the results of Fig. 2, a near-physiological concentration of dexamethasone (0·02 μM) was chosen for further *in vitro* experiments. NIH 3T3 cells were cultured and incubated with and without virus and dexamethasone in 24 well plates as described in Methods. After 72 h, the cells were harvested and prepared for flow cytometric quantification of virus-positive cells per well. There was no significant difference ($P=0·3$) between the number of wt FIS-2-positive cells and FIS-2 GRE mutant-positive cells when the cells were grown in the absence of dexamethasone (Fig. 4A). However, relatively higher levels of RT activity were detected in cultures infected with wt FIS-2 than with the FIS-2 GRE mutant: the RT activity per well was normalized relative to the value for FIS-2 GRE mutant-infected cells grown without dexamethasone in the same experiment (assigned a value of 1). An average 3·75 ± 0·33-fold higher RT activity was detected in wt FIS-2-infected cultures compared with FIS-2 GRE mutant-infected cultures (Fig. 4B). This observation indicated that more virus was produced from wt FIS-2-infected cells than from FIS-2 GRE mutant-infected cells.

In dexamethasone-supplemented cultures, there was a significant difference in the number of wt FIS-2-positive and FIS-2 GRE mutant-positive cells ($P=0·0003$). Dexamethasone increased the relative number of positive cells 5·2-fold for the FIS-2 GRE mutant and ninefold for wt FIS-2 (Fig. 4A). Some of the dexamethasone-induced increase in virus-positive cells could be a reflection of the positive effect of 0·02 μM dexamethasone on cell proliferation shown in Fig. 3(A). Dexamethasone augmented virus production only 1·4-fold in both wt FIS-2 and FIS-2 GRE mutant cultures, from 3·75 to 5·17 and from 1 to 1·35, respectively (Fig. 4B). However, when the cells were grown with a low serum (NCS) concentration, a more direct effect of dexamethasone on wt FIS-2 production was revealed: relative to the FIS-2 GRE mutant, RT activity in wt FIS-2-infected cultures grown without dexamethasone was reduced from 3·75 to 2·30 when the serum concentration in the medium was reduced from 10 % to 2·5 % (Fig. 4B, C). Addition of 0·02 μM dexamethasone together with wt FIS-2 resulted in a 2·7-fold increase (from 2·30 to 6·30) in virus production compared with virus production from wt FIS-2-inoculated cultures grown without dexamethasone (Fig. 4C). These results suggested that there were sufficient endogenous hormones present in medium supplemented

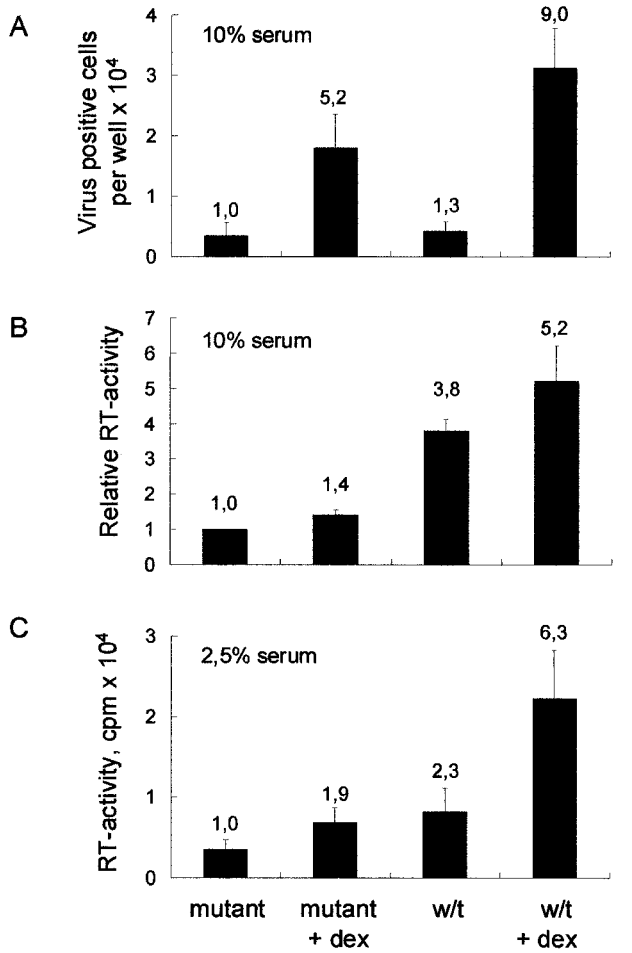

Fig. 4. (A) Flow cytometric quantification of virus-positive cells per well. Wild-type FIS-2 or FIS-2 GRE mutant-infected cells were grown without the addition of hormones or in the presence of 0·02 μM dexamethasone in medium supplemented with 10 % NCS. The cells were prepared for flow cytometry analysis at 72 h p.i. Uninfected NIH 3T3 cells served as controls. Each bar represents the average number of positive cells ± SD of 9 to 12 wells. Data were pooled from three independent experiments. (B) Relative RT activity in cultures of wt FIS-2 and FIS-2 GRE mutant-infected cells grown with or without 0·02 μM dexamethasone in medium supplemented with 10 % serum. Each bars represent average calculated relative levels from four independent experiments ± SD (see Fig. 2. for experimental design and representative example). (C) Effect of dexamethasone on virus production from *in vitro* virus-infected NIH 3T3 cells grown in medium supplemented with low serum concentration (2·5 % NCS). The data represent the average RT activity (c.p.m ± SD) from six wells. The numbers above each bar show calculated relative levels of RT activity when the FIS-2 GRE mutant was assigned a value of 1.

with 10 % serum to enhance the wt FIS-2 production and that addition of dexamethasone could augment virus production significantly when the cells were grown with a low serum concentration.

The glucocorticoid antagonist RU468 inhibits virus production in a dose-dependent manner

The type II glucocorticoid antagonist RU486–GR complex has been shown to bind weakly to the MMTV GRE and inhibit an agonist-induced transcription of the virus (Belikov *et al.*, 2001). We therefore used this antagonist to examine the involvement of GR and glucocorticoids in FIS-2 transcription. In addition to the synthetic glucocorticoid dexamethasone, we also stimulated the NIH 3T3 cells (cultured with 10 % serum) with the natural agonist hydrocortisone (cortisol). An initial hormone titration experiment showed that 0·2 μM hydrocortisone or dexamethasone augmented wt FIS-2 production on average 1·26 ± 0·2-fold and 1·33 ± 0·1-fold, respectively (72 h p.i., $n=3$). This concentration was therefore used for both agonists in subsequent experiments. The antagonist RU486 was added in 100-fold excess (20 μM) in order to display the agonists from GR.

In the first experiment, RT activity was assayed in the cell culture supernatant from wt FIS-2-infected NIH 3T3 cell cultures treated with different combinations of hormones and RU486. Cell cultures infected with the FIS-2 GRE mutant served as a reference. Concomitant addition of hormones and a 100-fold excess of RU486 resulted in a significant depression of wt FIS-2 production (Fig. 5A). When 20 μM RU486 was added alone, both wt FIS and the FIS-2 GRE mutant production were almost totally inhibited. However, when the cells were counted in a haemocytometer at the end of the experiment (116 h p.i.), we found that there were significantly lower cell numbers in RU486-treated cell cultures compared with cultures grown without the glucocorticoid antagonist ($P<0·01$). We then performed a second experiment where we assayed the effect of different RU486 concentrations (0–20 μM) on both cell proliferation and RT activity at 80 h p.i. with wt FIS-2 or the FIS-2 GRE mutant. The cells were grown with or without 0·2 μM hydrocortisone. In this experiment, the 20 μM RU496 dose also reduced the cell numbers significantly ($P<0·01$) (Fig. 5B). When we assayed the RT activity in the culture supernatants, we found that RU486 depressed virus production in a dose-dependent manner, also when the glucocorticoid antagonist did not inhibit cell proliferation significantly (Fig. 5C). Overall, these experiments indicated an involvement of GR in FIS-2 transcription.

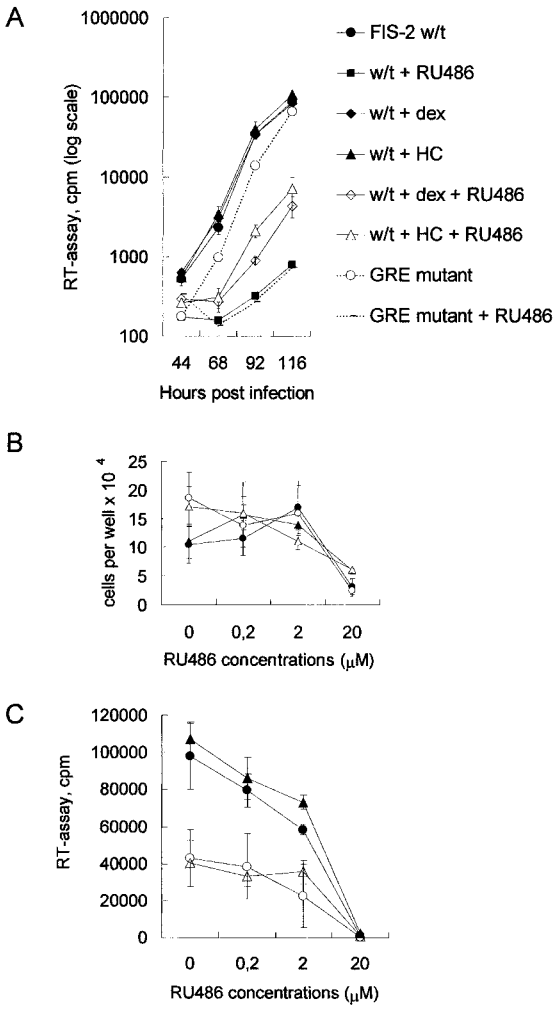

Fig. 5. Effect of the glucocorticoid antagonist RU486 on *in vitro*-infected NIH 3T3 cells. (A) RT activity at different times p.i. Each value represents the mean ± SD from six parallel experiments. The hormones were used at a concentration of 0·2 μM and the antagonist RU486 was used at a 100-fold excess (20 μM). dex, Dexamethasone; HC, hydrocortisone (cortisol). (B, C) Effect of different concentrations of RU486 on cell proliferation (B) and RT activity (C). Each point in (B) and (C) shows the mean ± SD from five parallel experiments at 80 h p.i. with wt FIS-2 (●), FIS-2 GRE mutant (○), wt + 0·2 μM hydrocortisone (▲) or GRE mutant + 0·2 μM hydrocortisone (△).

GRE is a major determinant of sex differences in susceptibility to FIS-2 infection

Repeated *in vitro* experiments have shown that the presence of an intact GRE can increase virus production and against this background we wanted to examine the functional significance of the GRE in the sex-related susceptibility to early FIS-2 infection. Young adult male and female mice were inoculated intraperitoneally (i.p.) with equal doses of either the mutated virus or the wt virus, and viraemia and virus titres in the spleens were assayed at different time points during primary infection. We observed a peak viraemia level at 8 days p.i. in male mice infected with wt FIS-2. Female mice infected with wt FIS-2 and male and female mice infected with the FIS-2 GRE mutant reached the peak viraemia level at 11 days p.i. (Fig. 6A).

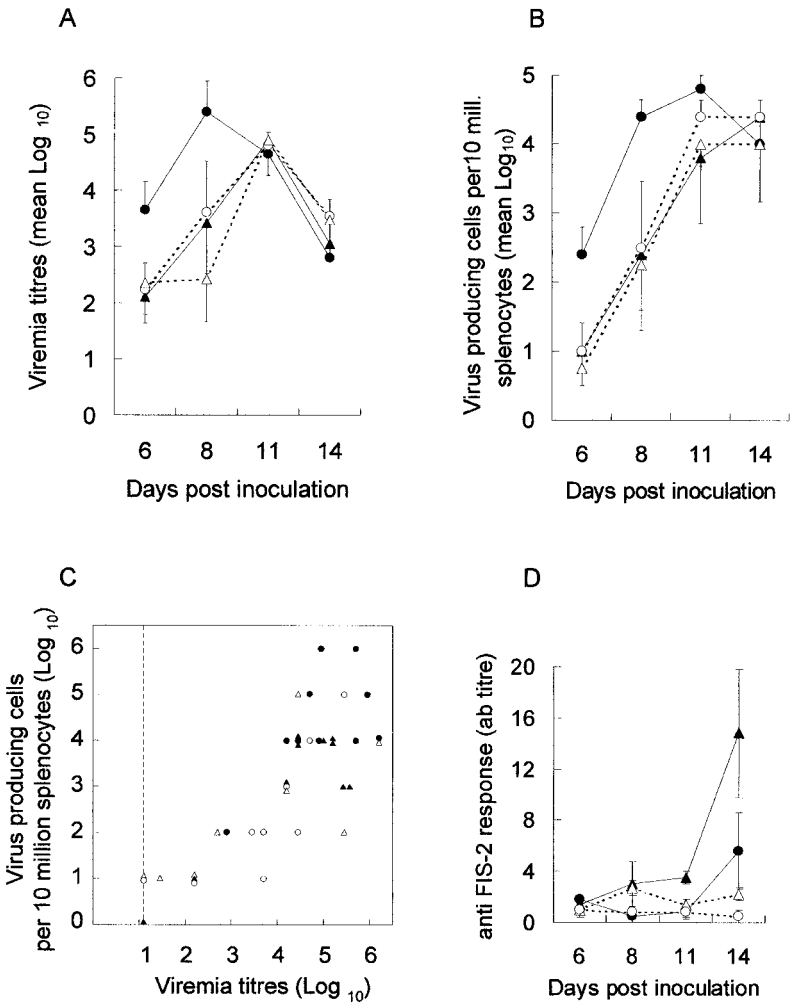

Fig. 6. *In vivo* studies during primary infection in male (●, ○) and female (▲, △) mice inoculated i.p. with $3{\cdot}6 \times 10^4$ IU of either wt FIS-2 (closed symbols) or FIS-2 GRE mutant (open symbols). Total number of mice in the experiments = 97. (A) Viraemia titres. Left censored values were given the value $\log_{10} = 1{\cdot}1$ (detection limit of the assay = 50 IU ml^{-1}) and each point represents the average viraemia titre from five mice. The bars indicate SEM. (B) Productively infected nucleated splenocytes during primary infection. Each point represents the average number of infected cells per 10^7 splenocytes ± SEM from five mice. (C) Correlation between viraemia titres and the number of virus-producing cells in the spleen at 8 days p.i. The dotted vertical line indicates the detection limit for the viraemia assay. Each point represents one mouse ($n = 9$–10 per group, total $n = 37$) and the data were pooled from two experiments. $R_s = 0{\cdot}707$ and $P < 0{\cdot}0001$. (D) Initial detection of antibodies directed against viral proteins on FIS-2-infected cells. Each point shows mean antibody titre ± SEM from five mice.

The primary target cells for both F-MuLV and FIS-2 are erythroid progenitors, while B and T cells are infected at a later stage of the infection (Bruland *et al.*, 2003). All of these cell types have steroid receptors present (Golde *et al.*, 1979; Hammond *et al.*, 1987; Miller *et al.*, 1998; von Lindern *et al.*, 1999). We therefore assayed the number of productively infected nucleated cells in the spleen (Fig. 6B). Average peak virus titres in the spleens from male mice infected with wt FIS-2 were detected at 11 days p.i. In accordance with the findings of our previous study (Bruland *et al.*, 2001), the wt FIS-2 dissemination was delayed in female mice. A comparable delay in virus spread was also observed in both female and male mice inoculated with the FIS-2 GRE mutant. The single point mutation in the GRE was stable after *in vivo* replication in male and female mice, as verified by RT-PCR and subsequent sequencing of the virus genome isolated from sera at 11 days p.i. (see Methods).

The difference in viraemia titres and numbers of virus-producing cells in the spleen between male mice inoculated with the wt FIS-2 and the other groups were most notable at 8 days p.i. The virus titres in serum and spleen at 8 days p.i. obtained in several experiments were therefore pooled and analysed statistically. Both viraemia titres ($n = 14$–22 per group, a total of 71 mice) and the number of infected cells in the spleen ($n = 9$–10 per group, a total of 39 mice) were significantly higher ($P < 0{\cdot}05$) in male mice infected with wt FIS-2 than in the three other groups at 8 days p.i. There was a strong correlation ($R_s = 0{\cdot}707$, $P < 0{\cdot}0001$) between viraemia titres and the number of virus-producing cells in the spleen (Fig. 6C). We found no significant difference in viraemia ($P = 0{\cdot}370$) or number of virus-positive cells in the spleen ($P = 0{\cdot}831$) between male and female mice infected by the FIS-2 GRE mutant. Hence, the observed sex difference in early FIS-2 dissemination was only significant when an intact GRE was present.

Viraemia titres were markedly reduced in all groups at 14 days p.i. (Fig. 6A). When we analysed for antibodies directed against viral proteins on FIS-2-infected cells, we found that the decline in viraemia titres at 14 days p.i. coincided with the onset of a detectable anti-FIS-2 response (Fig. 6D). The average anti-FIS-2 response in female mice was stronger than the average response in male mice at 14 days p.i. This is consistent with our previously reported observation that male mice are more immunosuppressed than female mice at 2 weeks p.i. when inoculated with equal

doses of wt FIS-2 (Bruland *et al.*, 2001). The levels of anti-FIS-2 response in both male and female mice inoculated with the FIS-2 GRE mutant were low, although female mice seemed to have slightly higher levels ($P=0{\cdot}05$).

DISCUSSION

The present study was undertaken to evaluate the contribution of the GRE in FIS-2 replication and to examine whether this element is a determinant for the sex differences observed in early FIS-2 infection (Bruland *et al.*, 2001). Our results from the *in vitro* virus infection of GR-containing NIH 3T3 cells indicated that both endogenous hormones in serum (NCS) and exogenous added glucocorticoid (dexamethasone) could enhance FIS-2 production when an intact GRE was present in the LTR U3 region. The wt FIS-2 and the FIS-2 GRE mutant seemed to infect cells with a comparable efficiency, but a higher RT activity was measured in the wt FIS-2 cultures than in FIS-2 GRE mutant-infected cultures when the cells were grown with a high serum concentration. The synthetic glucocorticoid dexamethasone augmented wt FIS-2 production significantly when the cells were grown with a low serum concentration. Additional experiments with glucocorticoid agonists (dexamethasone and hydrocortisone) and the glucocorticoid antagonist RU486 further indicated an involvement of the GR and GRE in FIS-2 replication. However, these results did not necessarily prove a functional significance of the presence of the GRE *in vivo*. The efficiency of GRE-mediated gene activation depends on several factors including the availability of receptors and hormones (Archer *et al.*, 1995; Gronemeyer, 1992; Nelson *et al.*, 1999, and references therein), and coactivator complexes and other transcription factors (Deroo & Archer, 2001). It was therefore of greater interest to compare replication of wt FIS-2 and the FIS-2 GRE mutant directly *in vivo*, i.e. in male and female adult mice. We then found that the wt FIS-2 disseminated faster than the FIS-2 GRE mutant in male mice and that the sex difference in early FIS-2 dissemination was only significant when an intact GRE was present. These results indicated that sex-related factors could activate virus gene transcription through the GRE.

A number of possible mechanisms may explain these observations. First, it is well documented that an androgen receptor and a progesterone receptor can bind to and activate a hormone response element with the common high-affinity core motif 5′-AGAACA-3′ found in the FIS-2 GRE (Fig. 1; Dai *et al.*, 1994). Nelson *et al.* (1999) suggested that this ‘one type fits all’ response element may be advantageous for extending the cellular host range of the virus to exploit a number of endocrine signalling pathways. This might be especially advantageous for the simple retroviruses whose host cells are limited to actively dividing cells (Lewis & Emerman, 1994). Erythroid progenitor cells are among the first cells infected in an FIS-2 infection, and the proliferation and differentiation of these cells are highly influenced by androgens and glucocorticoids (Udupa *et al.*, 1986; von Lindern *et al.*, 1999). Thus, androgens might affect virus production, both indirectly, through growth promotion of erythroblasts, and directly, through androgen receptor binding to the GRE in the FIS-2 LTR. Secondly, GRs in association with a chromatin-remodelling complex can bind to target sites within promoter regions of genes assembled as chromatin. This interaction alters the nucleosome architecture to allow binding of other transcription factors, such as NF1 (Fig. 1), that cannot bind an LTR assembled into chromatin (Archer *et al.*, 1992; for review on GR-mediated chromatin remodelling, see Deroo & Archer, 2001). One single GR binding site is sufficient for the structural transition to occur (Belikov *et al.*, 2000). The glucocorticoid antagonist RU486 promotes GR–GRE binding without inducing chromatin remodelling (Belikov *et al.*, 2001). In the present study we showed that RU486 inhibited FIS-2 production from *in vitro*-infected cells in a dose-dependent manner. This observation suggested that such a GR-induced chromatin remodelling might be important in FIS-2 transcription. It is possible that male mice have target cells with more or other factors that can influence a GR–GRE-mediated virus gene activation than female mice. Thirdly, young male mice in our study were more frequently involved in aggressive behaviour than female and castrated male mice (data not shown). This observation is in accordance with findings made by others, and it has been shown that behavioural factors such as testosterone-dependent aggression can modify the expression of sex differences in infection (reviewed in Klein, 2000; Azpiroz *et al.*, 2002). In our model system, we have observed that both male castrated mice and female mice are less susceptible to F-MuLV infection than non-castrated male mice (T. Bruland, L. A. S. Lavik, H. Y. Dai & A. Dalen, unpublished results). In mice, social conflict (i.e. inter-male aggression) increases the plasma corticosterone concentration (Dréau *et al.*, 1999; Padgett *et al.*, 1998). Since stress-induced hormones like cortisol bind to GR with high affinity, stress-induced elevating levels of circulating cortisol might have a stimulatory effect on FIS-2 production.

One should be cautious when drawing analogies from experiments with simple retroviruses like FIS-2 to complex retroviruses such as HIV, due to their production of accessory proteins and their ability to infect non-dividing cells (Coffin *et al.*, 1997; Lewis & Emerman, 1994). However, there are some interesting resemblances in relation to hormonal influence on virus production: the HIV provirus does contain an intact and functional GRE within the *vif* open reading frame (Soudeyns *et al.*, 1993). The HIV-1 virion-associated accessory protein, Vpr, has also been shown to be a co-activator of the human GR (Kino *et al.*, 1999; Refaeli *et al.*, 1995) and it has been suggested that an intact GRE in the HIV-1 LTR is critical for Vpr activity (Sherman *et al.*, 2000; Vanitharani *et al.*, 2001). Glucocorticoids have been shown to both activate (Kolesnitchenko & Snart, 1992; Russo *et al.*, 1999) and suppress (Kino *et al.*, 2000; Mitra *et al.*, 1995; Russo *et al.*, 1999) HIV replication *in vitro*. The functional significance of

the presence of the GRE in *in vivo* HIV replication is still uncertain. However, in the context of our findings, it is intriguing that several studies have shown that women have lower numbers of circulating HIV RNA copies than men (reviewed in Gandhi *et al.*, 2002), particularly at or near the time of seroconversion (Evans *et al.*, 1997; Lyles *et al.*, 1999; Sterling *et al.*, 1999).

In conclusion, our results in this report show that the presence of a single intact GRE in FIS-2 U3 can enhance virus production from *in vitro*-infected NIH 3T3 cells. In addition we have shown that sex-related factors can influence early FIS-2 replication *in vivo* via mechanisms involving this element. The exact mechanisms for sex-related virus production through the GRE require further examination. Work in progress is now focusing on possible stress- and/or androgen-induced virus gene activation.

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