Novel retroviral sequences are expressed in the epididymis and uterus of Syrian hamsters

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Combined androgen and oestrogen treatment of male or female Syrian hamsters results via an unknown mechanism in the formation of leiomyosarcomas in the reproductive tract. We have examined the possibility that retroviral gene expression may play a role in tumorigenesis. Evidence of virus-like particles in epididymis and seminal fluid is shown in electron micrographs. We identified expressed retroviral sequences by using RT–PCR to amplify a conserved retroviral reverse transcriptase coding region in RNA isolated from epididymis, testis, clarified seminal fluid and uterus. Phylogenetic analysis allowed us to classify the sequences into two distinct groups: (1) mammalian type-C viruses, having similarity to Moloney murine leukemia virus, feline leukaemia virus and gibbon ape leukemia virus amongst others; (2) a mixed ABCD group containing, for example, Chinese hamster and murine intracisternal A-particle virus sequences, mouse mammary tumour virus and human and simian retroviral sequences. The presence of putative full-length retrovirus related to mammalian type-C viruses in the epididymis and uterus was confirmed by Northern blot analysis. However, steroid treatment did not alter retroviral RNA levels in the epididymis or in a uterine tumour relative to untreated uterus. In summary, Syrian hamster reproductive tissues were found to express unique retroviral sequences; however, their role, if any, in hormonal carcinogenesis remains unresolved.

Introduction

Syrian hamsters (Mesocricetus auratus) have an increased incidence of spontaneous tumour formation in aged animals. However, a number of tissues, including the kidney, liver, vas deferens and uterus, demonstrate unusual susceptibility to neoplastic transformation after administration of steroid hormones (reviewed in Kirkman, 1972). The majority of tumours induced in the vas deferens and uterus are leiomyosarcomas (Kirkman & Algard, 1965, 1970). In the male, the earliest tumours form in the smooth musculature at the epididymis/vas deferens junction and later tumours arise further down the vas deferens.

Although the mechanism of leiomyosarcoma induction is unknown, there is an obligatory requirement for administration of both androgen and oestrogen for periods of 300–400 days. Due to the length of time required for tumour formation, both transplantable tumours and cloned cell lines were developed to facilitate studies of the mechanism of tumour induction. We have published extensively on the vas deferens-derived DDT, MF2 cell line (Cornett & Norris, 1982; Norris et al., 1974, 1984, 1987, 1991; Smith et al., 1984; Syms et al., 1984). These cells have androgen, glucocorticoid and α₁- and β₂-adrenergic receptors, and maintain many of the features characteristic of their smooth muscle origin. DDT, MF2 cell growth in vitro is increased by androgens whereas glucocorticoids block growth in the G₀/G₁ phase of the cell cycle. This blockage can be overcome by the administration of platelet-derived growth factor (PDGF) or fibroblast growth factor, but not epidermal growth factor or insulin. These observations suggested an autocrine regulation of tumour cell growth and led to studies devoted to cloning and identifying

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putative autocrine growth factors. Screening of a DDT<sub>1</sub>MF2 cDNA library with the v-sis oncogene resulted in identification and cloning of a recombined Simian sarcoma virus-related sequence that contained an intact PDGF-β chain (Harris et al., 1992). The expression of a retroviral sequence containing a growth factor gene suggested a mechanism by which androgens and oestrogens might cause tumours in the Syrian hamster reproductive tract: steroid treatment may affect expression or recombination of endogenous retroviruses with a concomitant alteration in oncogenic potential.

Expression of endogenous retroviruses and retrovirus-like elements, and their potential role in carcinogenesis, have been studied extensively in many species (Asch et al., 1993; Weiss et al., 1982, 1985), and the expression of some retroviruses is increased following treatment with androgens, oestrogens or progestins (Cato et al., 1987; Cornell et al., 1992; Ono et al., 1987). Several Syrian hamster tumours and tumour-derived cell lines have been shown to contain retrovirus particles and reverse transcriptase activity (Dodge, 1974; Gregerson et al., 1989; Russell et al., 1979). In addition, the Syrian hamster genome is estimated to contain 800–950 copies of intracisternal A-particle (IAP) elements per haploid genome (Lueders & Kuff, 1983; Suzuki et al., 1982). Although proviral IAP sequences have been cloned from the Syrian hamster genome (Lueders & Kuff, 1983; Ono et al., 1985; Suzuki et al., 1982), expressed retroviral sequences in normal Syrian hamster tissues have not been cloned. Thus, to begin to investigate the potential role of retroviral expression in steroid-induced carcinogenesis, we sought and now report detection of expressed retroviral sequences in the Syrian hamster reproductive tract.

Methods

**Animals and steroid treatment.** Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA) and housed by the Division of Animal Resources at the Medical University of South Carolina (fully accredited by the American Association for Accreditation of Animal Laboratory Care). For steroid treatment, hamsters were implanted with two steroid pellets containing testosterone propionate of Animal Laboratory Care). For steroid treatment, hamsters were implanted with two steroid pellets containing testosterone propionate (Cato et al., 1987). Animals and steroid treatment.

**RNA purification.** Total RNA from hamster tissues was isolated by a guanidinium isothiocyanate procedure (Chomczynski & Sacchi, 1987). Epididymal semen from young males was collected as described (Kissling et al., 1987), clarified by low-speed centrifugation and filtration (0.45 μm), and potential virus particles were pelleted at 95 000 g for 90 min. RNA was extracted from the pellet by the method described above. All RNA samples were treated with RNAse-free DNase I (Boehringer Mannheim) to remove contaminating DNA.

**cDNA Synthesis and PCR.** The methods used to detect and clone expressed retroviral sequences are a synthesis of two previously described methods (Donehower et al., 1990; Shih et al., 1989). Total cellular RNA (50–60 μg) or virus pellet RNA (1–2 μg) was reverse transcribed using M-MLV RNase H reverse transcriptase (Superscript, GibcoBRL) and either oligo(dT), or random hexanucleotide primers. Following cDNA synthesis, RNA was removed by alkaline hydrolysis. Template cDNA (one-fifth of the cDNA preparation) was heat-denatured at 95 °C for 5 min prior to PCR amplification. PCR was performed with Taq polymerase according to the GeneAmp PCR procedure (Perkin-Elmer/Cetus) with the following programme: denaturation at 94°C for 1 min, annealing at 55 °C for 30 s, extension at 72 °C for 1 min for 24 cycles followed by a final extension of 3 min. Degenerate PCR primers were those described by Shih et al. (1989):

- 5′ Primer: 5′ TGGAAAGTG(C/T)G(A/C)(A/G)GA
- 3′ Primer: 5′ G(G/C)A(GG)CAGCACGC(A/G)/TGTCACTCAAG(T/C)GTA

Oligonucleotide primers were synthesized in the Biochemistry Department of the Medical University of South Carolina with an Applied Biosystems 380A Synthesizer.

To confirm that RNA preparations did not contain DNA contamination, an aliquot of RNA from each sample was subjected to PCR prior to reverse transcription and analysed for amplified products. Negative samples were used for subsequent cDNA synthesis and PCR amplification. Other negative PCR controls were cDNA samples amplified without primers, and PCR mixtures containing primers but no cDNA. PCR products were fractionated by PAGE and visualized by ethidium bromide staining.

**Molecular cloning and sequencing.** PCR-generated DNA was eluted from polyacrylamide gel slices, ligated to Smal- or EcoRV-restricted pBluescript II SK(−) (Stratagene) and selected by blue/white screening. Plasmids containing inserts were sequenced with Sequenase (Amersham) cDNA Synthesis and PCR.

**Northern blotting.** Total RNA was run on a 1% agarose/3:7% formaldehyde gel, transferred to a Nytran membrane (Schleicher & Schuell) and UV cross-linked. Membranes were probed individually with a representative of each group listed in Fig. 2 (notation: 1, 2, 3, 4) with a [32P]-UTP-labelled riboprobe (following protocol by Stratagene) in 5 × SSPE, 25 mM Tris–HCl (pH 7.5), 0.1% sodium pyrophosphate, 1% SDS, 0.2% Ficoll, 0.2% polyvinylpyrrolidone and 5 mM EDTA (pH 8). Membranes were hybridized at 65 °C and washed at a final stringency of 0.5 × SSPE, 0.025% SDS at 65 °C. Radioactive signals were analysed on a PhosphorImager (Molecular Dynamics). A [32P]-UTP-labelled riboprobe generated from a murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to assess RNA loading. Figures were prepared with Adobe Photoshop (Adobe Systems) from images generated by the PhosphorImager.

**Electron microscopy.** Epididymal semen and epididymis from untreated animals were removed and placed directly into PBS containing 1% (w/v) paraformaldehyde and 2% (w/v) glutaraldehyde. Epididymides were cut into three anatomical sections (caput, corpus and cauda). The epididymal semen was pelleted at 3500 g for 10 min after 24 h fixation, and both the tissues and pellet were rinsed in 0.2 M sodium cacodylate buffer, post-fixed with 2% (w/v) osmium tetroxide and 3% (w/v) potassium ferrocyanide, and rinsed with 0.2 M sodium cacodylate buffer, prior to routine dehydration with graded alcohols and propylene oxide. Samples were embedded in Epon–Araldite 502 resin, sectioned (100 nm), and examined with a JEOL 100S or JEOL 1200EX electron microscope (Department of Pathology and Laboratory Medicine, Medical University of South Carolina).
Retroviral expression in Syrian hamsters

Fig. 1. Electron micrographs demonstrating retrovirus-like particles in Syrian hamster tissues. (A) C-type virus particles in epithelial cell of caudal epididymis, as indicated by arrowheads. (B) D-type virus particle in seminal fluid, as indicated by an arrowhead. Bars, 500 nm (inset bars, 100 nm).

Phylogenetic analyses. The sequences analysed (e8, e14, e18, e22, e23, e26, s1, s12, s19, s25, t2, t13, t12, ut3, ut10 and ut16) were derived from the epididymis (e), seminal fluid (s), testis (t) and uterus (ut). The other sequences were obtained from GenBank (release 88) using the Blast program. Phylogenetic analysis of the 90 bp amplified fragment from each tissue began with a GCG (v 9.1) PileUp alignment. The alignments of the sequences were then re-computed by CLUSTAL W (v 1.73) (Thompson et al., 1994). The Jukes–Cantor (JC) distances computed on the CLUSTAL W alignment for the combined set of 33 sequences (all sequences in Fig. 2) ranged from 3 to 155 substitutions per 100 bp. Of the pairwise comparisons, 435 of 528 gave JC distances between 30 and 100 while 47 of 528 gave distances greater than 100. Based on this information, the 33 sequences were separated into two groups: mammalian type-C and mixed ABCD. The CLUSTAL W alignments of each new group were analysed for phylogenetic relationships by a distance method (DNADIST/FITCH) and the reliability of groupings was tested by bootstrap analysis (PHYLIPI version 3.57c; Felsenstein, 1993). The plots are labelled where the bootstrap support was above 70/100.

Nucleotide sequence accession numbers. Reference sequences used as representative members of the mammalian type-C virus and mixed ABCD group are listed in the following format: name (abbreviation), GenBank accession number(s) (reference). Moloney murine leukaemia virus (m-mlv), J02255, J02256, J02257 (Shinnick et al., 1981); AKV murine leukaemia virus (akv-mlv), J01998 and following (Herr, 1984); Friend murine leukaemia virus (f-mlv), M93134, M81185 (Masuda et al., 1992); chimpanzee endogenous retroviral DNA (chpinorc), M69088 (Shih et al., 1991); baboon endogenous virus (rebevxx), X05470 (Kato et al., 1987); feline leukaemia virus (felv), M18427, M19392 (Donahue et al., 1988); Syrian hamster IAP virus (sh-iap), M10134, K02288, K02289 (Ono et al., 1985); Chinese hamster IAP-related sequence (ch-iap-r), M73970 (Dorner et al., 1991); mouse IAP virus (miap), M17551, K01572, K01573 (Mietz et al., 1987); mouse mammary tumour virus (mmtv), M15122 (Moore et al., 1987); simian SRV-1 type D retrovirus (srv-1), M11841 (Power et al., 1986); simian SRV-2 type D retrovirus (srv-2), M16605 (Thayer et al., 1987); simian sarcoma virus (ssv), M23385 (Oda et al., 1988); Jaagsiekte sheep retrovirus (jsr), M80216 (York et al., 1992); human endogenous retrovirus (herv-k10), M14123 (Ono et al., 1986); human mmtv-related sequence (hm-16), M30520 (Deen & Sweet, 1986); Rous sarcoma virus (rsv), J02342, J02021, J02343 (Schwartz et al., 1983).

Results

Evidence of retrovirus-like particles and detection of conserved reverse transcriptase coding domains in Syrian hamster tissues and seminal fluid

Electron microscopic evidence of retrovirus-like particles is shown in Fig. 1. C-type particles were seen budding from the epithelial lining of the caudal epididymis (Fig. 1A). Also,
A PCR approach (Donehower et al., 1990; Shih et al., 1989) was used to amplify conserved amino-terminal reverse transcriptase (pol) coding regions of cDNA derived from diaphragm, epididymis, testis, seminal fluid and uterus. The PCR primers used were mixed oligonucleotide primers (MOP) described by Shih et al. (1989), and are based on reverse transcriptases of 18 retroviruses or retrovirus-like genes of various origins including human, monkey, baboon, mouse, chicken, cow and horse. Examination of more than 50 PCR products by PAGE demonstrated DNA fragments of the expected 138 bp size, as well as larger and smaller fragments, from each source except diaphragm. No products were found in negative controls. Because no products were found in cDNA from diaphragm, this tissue was used as a negative control in subsequent experiments. The PCR-generated DNA fragments were cloned and sequenced. Many of the clones were found to contain the same nucleotide sequence and the 16 clones reported here are representative of the spectrum of sequences obtained. All but one (ut10) of the sequences isolated from the uterus were related to the mammalian type-C group. All but two seminal fluid isolates were related to mixed ABCD members. One testis isolate, clone t12, was of the expected 138 bp size. Clone t2 had the expected 91 bp amplified fragment but a truncated 3′ primer. Clone t3 had a 1 bp insertion at position 1 in the amplified fragment. All other sequences were of the expected 138 bp size.

**Comparison of amplified sequences with known reverse transcriptase genes**

Each clone was compared with all sequences present in GenBank (release 88). Only bp 2–91 of the amplified region, corresponding to the open reading frame for protein translation, were considered in comparisons. In the mammalian type-C group clones e14 and t2 are 75%, ut16 is 74%, e26 is 73% and ut3 is 68% identical to m-mlv at the nucleic acid level. Additionally, clones e14, t2 and e26 are 90%, clone ut16 is 87%, and clone ut3 is 77% identical to m-mlv at the deduced protein level (Figs 2 and 3). Clones e8, e18, e22, e23, s1, s12, s19, s25, t3, t12 and ut10 were most identical to members of the mixed ABCD group (Figs 2 and 3). However, the sequence identities of the mixed ABCD group at the nucleic acid and deduced protein levels were not as robust as those for the mammalian type-C virus group. For example, clone e8 is 63% identical to sh-iap at the nucleic acid level but only 50% identical to sh-iap at the deduced protein level.

**Phylogenetic analysis of amplified sequences**

To simplify the phylogenetic analysis, only representative clones from this study and members of the mammalian type-C
Retroviral expression in Syrian hamsters

Mammalian type-C Group

| m-mlv       | FKNSPLFDE ALHRLDADF IQHPDLILLQ |
| f-mlv       | .................................. |
| chpinorfc   | .................................. |
| felv        | .................................. |
| e14         | .................................. |
| t2          | .................................. |
| ut16        | .................................. |
| e26         | .................................. |
| ut3         | .................................. |

Mixed ABCD Group

| sh-lap      | MANSPICQI YQYEALPEIR KQFTSLIVIH |
| ch-lap-r    | .................................. |
| m-lap       | .................... M ... M ........ L.V ... E.P ... LLL |
| e8          | LSD ... L.Y F.KP.I.H ... PQS.IY. |
| s12         | LSD ... L.Y F.KP.I.H ... PQS.IY. |
| t12         | LSD ... L.Y F.KP.I.H ... PQS.IY. |
| s25         | L.G ... L.D F.KP.I.P.H ... L.Y |
| e22         | L ... L.Y F.KP.I.P.H ... L.Y |
| etc         | .................................. |
| mntv        | K ... L.K F.DK.ILTV. DQYQDSYIV |
| srv-1       | L ... L.K AT.IHKV. HAWQMYI. |
| srv-2       | L ... L.K AA.I.V. SWAQMYI. |
| srcv        | .................................. |
| jsv         | .................................. |
| hery-k10    | L ... L.T F.GR.Q.V. EK.SDYY |
| hm-16       | L ... L.T F.AQ.G.VQ. DK.SDYY |
| rsv         | TG ... V.GOV.L ... LHRP. CML |
| e18         | .................................. |
| s1          | .................................. |
| s19         | .................................. |
| t3          | .................................. |
| ut10        | .................................. |

Fig. 3. Deduced protein sequence alignment of mammalian type-C and mixed ABCD group pol sequences. Amino acid changes are indicated with reference to the individual sequence heading each group. Identities are indicated by a dot.

A lower level of expression was observed in the kidney. Diaphragm was used as a negative control. Although no expression was detected in the testis when total RNA was used, a low level of expression was found using poly(A)+ selected RNA (not shown). Since Northern blots did not detect RNA expression from the mixed ABCD group, we used the increased sensitivity of nuclease S1 protection analysis (data not shown). Briefly, with 3 µg of poly(A)+ selected RNA from epididymis and 32P end-labelled probes, only hamster type-C pol expression was detected. Northern blots and nuclease S1 protection assays were used to evaluate the effect of steroid treatment on the hamster type-C virus expression. Although different levels of hamster type-C virus RNA expression in control as well as steroid-treated animals were noted, there was no consistent alteration in expression due to steroid treatment in any tissues examined. However, we did find hamster type-C virus RNA expression in primary uterine tumour tissue but not in primary vas deferens tumour tissue (Fig. 5).

Discussion

The identification of retroviral sequences by PCR, detection of their expression in testis, epididymis, uterus and seminal fluid by Northern blot analysis, and electron micrographic evidence of virus-like particles in epididymis and seminal fluid, show the Syrianhamster reproductive tract to be a site of retroviral expression. These observations are similar to earlier studies on mice (Kiessling et al., 1987, 1989) and provide additional evidence for the presence of retroviruses in rodent reproductive organs.

Phylogenetic analysis groups our sequences with the mammalian type-C and mixed ABCD groups. The JC distances computed from the CLUSTAL W alignment of the 33 sequences were in all likelihood under-estimates of the intersequence distances, due to the conservative estimate of the number of repeat substitutions per site coupled with the short sequences used. Li et al. (1995) reported that the sequence identity, based on the entire length of pol, between the mixed ABCD group and mammalian type-C was approximately 50%. Therefore, the trees constructed from the short (90 bp) sequences from this survey are not reliable as estimates of the relatedness of entire pol sequences. Protein parsimony (PROTPARS) and DNA maximum likelihood (FASTDNAML) analyses were also performed on the CLUSTAL W alignments of the mammalian type-C and mixed ABCD groups, but gave similar results to DNADIST/FITCH (data not shown). Even though the phylogenetic trees were unreliable when analysing the 90 bp sequences, our data suggest that the Syrian hamster clones in the mammalian type-C and mixed ABCD groups represent new group members.

Although we did not detect expression of the mixed ABCD group by either Northern blot or nuclease S1 analysis, Southern blot analysis (not shown) using e22, s1 and s12 separately as probes confirmed that the sequences are present in the Syrian...
Fig. 4. Phylogenetic trees (DNADIST/FITCH) of pol clones based on nucleotide sequences from epididymis (e), seminal fluid (s), testis (t) and uterus (ut) and previously described pol sequences (see Methods for description). The mammalian type-C group is represented in (A) and the mixed ABCD group is represented in (B). Branch lengths are proportional to sequence divergence and all bootstrap values greater than 70/100 are shown at branch points for each tree.

Fig. 5. Northern blot analysis of 5 µg total RNA from Syrian hamsters. Control animals (C) were an 11-month-old male (kidney, diaphragm, testis and epididymis) and an 8-month-old female (uterus). Hormone-treated animals (TP/DES) were a 13-month-old male (kidney, testis, epididymis and vas deferens tumour) and an 8-month-old female (uterine tumour). The blot was probed with a riboprobe (e14) from the mammalian type-C group. Arrow indicates a putative full-length retrovirus approximately 8.5 kbp in length.

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Hamster genome. Furthermore, we found these sequences to be hamster-specific (i.e. not present in mouse, rat or human DNA) but much less prevalent than the 800–950 copies of the Syrian hamster proviral IAP sequence (Lueders & Kuff, 1983; Suzuki et al., 1982).

The involvement of retrovirus expression in carcinogenesis is best illustrated by the MMTV induction of mouse mammary tumours (Dickson, 1990; Nusse, 1990), whereby retrovirus integration into preferred sites of the genome alters transcriptional mechanisms in cis and leads to the activation of adjacent proto-oncogenes (wt genes). The influence of sex steroids on MMTV carcinogenesis is uncertain. However, a provirus whose expression is androgen-dependent and is predominantly expressed in the murine reproductive tract has been identified (Cornwall et al., 1992). Our results show no consistent alteration in hamster type-C pol expression during steroid treatment. Thus, with the current evidence, the role of retroviral expression in hormonal carcinogenesis in the Syrian hamster reproductive tract remains inconclusive.

We have used a sensitive PCR assay to demonstrate retroviral gene expression in the male and female Syrian hamster reproductive tracts. Furthermore, we have found that hamster type-C pol sequences and a putative full-length retrovirus are primarily expressed in the epididymis and uterus, with a lower level of expression in kidney and testis. However, because the animals appear healthy and reproduce without problems, the biological significance of retroviral expression is uncertain. Our results suggest that, like in mice (Kiessling et al., 1987) and humans (Medstrand & Blomberg, 1993), expression of endogenous retroviral sequences may occur without any obvious pathological effects.
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References


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