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Mouse mammary tumour virus related sequences are present in human DNA

Felicity E.B.May, Bruce R.Westley and Henri Rochefort
Unité d'Endocrinologie Cellulaire et Moléculaire, INSERM. (U 148), 60, Rue de Navacelles, 34100 Montpellier, France

Elena Buetti and Heidi Diggelmann
Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Switzerland

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ABSTRACT

MuMTV-related sequences have been identified in the DNA of human breast cancer cells using the Southern transfer technique and hybridisation with cloned MuMTV DNA under conditions in which partially mismatched sequences form stable hybrids. Hybridisation with cloned fragments of the MuMTV genome showed that the gag-pol region shares the most homology (estimated to be >80%) with the human MuMTV-related sequences, however, DNA fragments partially homologous to the MuMTV LTR, gag and env regions were also detected. Analysis of several human DNA samples suggests that the majority of the human MuMTV-related sequences are genetically transmitted but additional Eco R1 fragments were detected in the DNA of one out of three breast cancer cell lines, MCF$_7$. These sequences are potential probes for the human MuMTV-related retroviral sequences and will allow their possible role in human breast cancer to be evaluated.

INTRODUCTION

The mechanism of human mammary carcinogenesis is not understood even though the role of steroid hormones, in particular oestrogens as tumour promoting agents is well established (1). In susceptible mice strains, two agents appear to act synergistically to induce mammary tumours ; murine mammary tumour virus (MuMTV) a B-type retrovirus (2) and ovarian hormones. While the growth of murine mammary tumours becomes unresponsive to ovarian hormones, one third of human breast cancers remain oestrogen responsive. Murine mammary tumour cells, however, remain responsive to glucocorticoids since MuMTV expression is regulated in vitro by glucocorticoids (3), probably through an interaction of the glucocorticoid-receptor complex with a sequence within the long terminal repeat (LTR) (4,5).

Expression of two MuMTV proviruses (from either MTV-1 or MTV-2) results in a high incidence of mammary tumours (6) while others are implicated in low-incidence, late-onset mammary carcinogenesis (7). MuMTV proviral copies inherited via the germline are present in the DNA of most mouse cells (endogenous transmission), but mammary tumour cell DNA may contain additional proviral copies integrated as a result of MuMTV infection (exogenous transmission) (8). Recently, closely related transforming genes have been identified by DNA transfection experiments in the MCF_7 human mammary cancer cell line and in mouse mammary carcinomas which were originally transformed by MuMTV (9). This suggests that common mechanisms of tumourigenesis may be involved.

Epidemiological, immunological and biochemical studies suggest that a retrovirus may be involved in the aetiology of human breast cancer (1). This view, however, remains controversial ; the validity of many studies has been questioned and the "retroviral particles" have proved elusive (10). B-type retroviral-like particles (11) containing reverse transcriptase activity (12) have been identified in human milk samples and MuMTV-related RNA (13,14) and antigens (15) were subsequently reported in some human tumours. Two human breast cancer cell lines (T-47D (16,17) and early passages of MCF_7 (18,19) have been reported to release retrovirus-like particles. In these cell lines, which contain oestrogen receptors, the synthesis of specific proteins is regulated by oestrogens (20). Their proliferation is also stimulated by oestradiol (21,22) as is the growth of $\simeq$ 33% of human mammary carcinomas. These cell lines are therefore good potential systems with which to study the role of oestrogen and viral sequences in human mammary carcinogenesis.

If an MuMTV-like retrovirus has a role in human mammary tumourogenesis, proviral DNA whether endogenously or exogenously transmitted should be present in human breast tumour DNA. Previous reports using uncloned MuMTV cDNA and solution hybridisation have failed to provide convincing evidence for MuMTV-related proviral sequences in human DNA because of the extremely low levels of hybridisation (23,24).

To provide more tangible evidence for the existence of such a retrovirus, we have used cloned probes and the more sensitive Southern transfer technique (25) to investigate whether proviral copies of a virus related to the murine mammary tumour virus are present in human DNA.

MATERIALS AND METHODS

Preparation and Fractionation of DNA

The human cells were cultured as described previously (20). Mouse GR cells were maintained in Dulbecco's modified Eagles medium containing 5% foetal calf serum. DNA was prepared using established methods and digested to completion with Eco R1. After electrophoresis of 10 µg aliquots on 1% agarose gels (26), DNA was stained with ethidium bromide, photographed and transferred to nitrocellulose (25).

^{32}P-Labelled Probes

The ≃9 kb permuted MuMTV insert of the λ clone I prepared from closed circular MuMTV DNA of HTC-MI cells (27) was excised with Eco R1 and purified from the λ arms on sucrose gradients. Cloned MuMTV Pst 1 fragments (28,29) were purified from the vector (pBR 322) DNA after Pst 1 digestion, by agarose gel electrophoresis (30). DNA was labelled with ^{32}P by nick-translation (31) to a specific activity of 2×10^8 cpm/µg.

Hybridisation and Washing Conditions

Hybridisation was in 15-35% formamide, 0.6M NaCl, 20 mM EDTA, 200 mM Tris pH 8.0, 5 x Denhardts solution (32), 0.1% sodium pyrophosphate, 0.2% SDS, 250 µg/ml yeast RNA, 100 µg/ml salmon sperm DNA and 2×10^6 cpm/ml of alkali-denatured ^{32}P-labelled probe for 90 h at 37°C. After hybridisation, the filters were washed 2 times for 1 h in the percentage of formamide present during hybridisation (15-35%), 5 x SSC (1 x SSC : 150 mM NaCl, 15 mM sodium citrate), 10 µg/ml calf thymus DNA, 0.1% sodium pyrophosphate and 0.2% SDS at 37°C, then 3 times for 30 min. in 3 x SSC, 10 µg/ml calf thymus DNA, 0.1% sodium pyrophosphate and 0.2% SDS, blotted dry and exposed to preflashed Kodak XS X-ray film against a fast tungstate intensifying screen.

RESULTS

MuMTV DNA containing the entire viral genome of a GR-MuMTV provirus (see Fig. 1) was cloned from unintegrated circular DNA (27), purified from vector DNA and hybridised to Southern transfers of Eco R1 digested DNA from T-47D cells and GR mouse cells under standard conditions used to detect homologous DNA sequences (50% formamide, 4 x SET at 37°C (30)). Hybridisation to DNA from the GR mouse cell line, which contains 40-50 additional copies of integrated GR proviral MuMTV DNA per genome (28) as a result of exogenous infection, revealed multiple fragments of >4.0 kb (data not shown) which are determined by the unique proviral Eco R1 site and the Eco R1 sites in the flanking mouse DNA (Fig. 1). Under these conditions, no hybridisation to T-47D DNA was detected (data not shown).

The stringency of the hybridisation conditions was therefore lowered by decreasing the formamide concentration in the hybridisation solution to permit detection of non homologous MuMTV-related sequences. Fig. 2 shows the patterns obtained when MuMTV DNA was hybridised at various stringencies to Eco R1 digested DNA from GR cells and T-47D cells. The extent of hybridisation to the multiple homologous MuMTV Eco R1 fragments in GR DNA was similar at each stringency although the background was higher after hybridisation in 15% formamide. Longer exposure of the filters (not shown) revealed additional fragments which

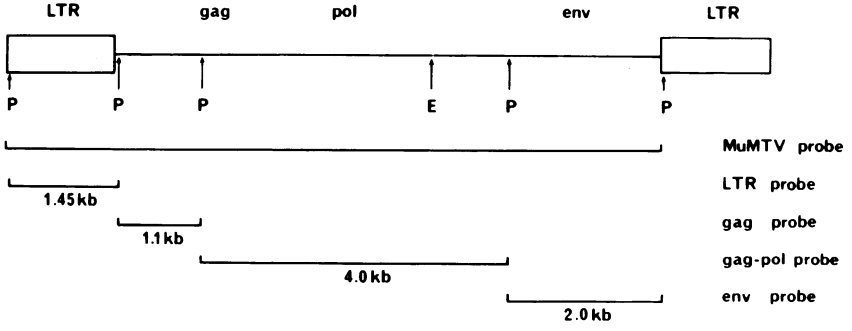

Figure 1. Schematic representation of the GR MuMTV provirus.
The Pst 1 (P) and Eco R1 (E) sites within the provirus are indicated and the position of all the fragments (27,28,29) used for hybridization in this study are shown below.

are presumably the recently described MuMTV-β sequences (33) as they only hybridised under conditions of lowered stringency. Hybridisation to discrete T-47D Eco R1 fragments (Fig. 2) in the presence of 15% formamide (the lowest stringency tested) was obscured by a high level of background. In the presence of 20 and 25% formamide, the background was lower and hybridisation to discrete Eco R1 fragments of 1.0, 2.1, 3.1, ≈3.8, 5.2 and 7.5 kb was visible. Hybridisation was much weaker, but was visible on the original autoradiogram, at 30% and was undetectable at 35% formamide. MuMTV was also hybridised to DNA prepared from a

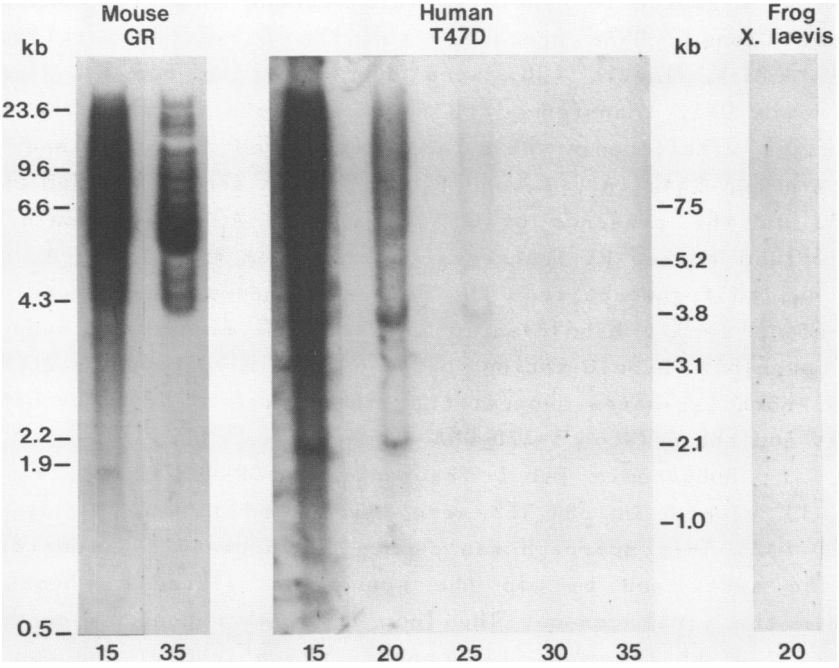

Figure 2. Detection of MuMTV-related sequences in T-47D cell DNA. Eco R1 digested DNA from GR cells, T-47D cells and X. laevis liver was separated on agarose gels, transferred to nitrocellulose and hybridised to nick-translated MuMTV DNA (MuMTV probe, Fig. 1) as described in the Materials and Methods. The percentage of formamide contained in the hybridisation solution is indicated below each track. λ DNA digested with Hind III was used as the molecular weight marker. The positions of the marker fragments are indicated on the left of the figure and of the T-47D Eco R1 fragments which hybridised with MuMTV on the right.

nonmammalian vertebrate (the frog, Xenopus laevis). No hybridisation was detected in the presence of 20% formamide (Fig. 2).

The degree of homology of two related sequences may be estimated from the Tm of their heteroduplex (1% mismatch lowers the Tm by ≃ 0.7°C) (34). A change of 1% in the formamide concentration alters the Tm of a DNA duplex by 0.72°C (35). The decrease in the formamide concentration which was required for MuMTV DNA to hybridise to fragments of human DNA suggests that these sequences are ≃13% mismatched. The degree of homology between MuMTV DNA and the related human sequences was also evaluated experimentally by determining the ability of sequences of known mismatch to hybridise at different formamide concentrations. Cloned cDNAs prepared from the 4 related vitellogenin mRNAs of X. laevis (36) were hybridised to Eco R1 digested X. laevis DNA, transferred to nitrocellulose filters. The A and B group vitellogenin RNAs are mismatched by ≃ 20% and both contain two RNAs (A_1, A_2 and B_1, B_2) which are mismatched by ≃ 5% (36). In the presence of 25% formamide, A_1 vitellogenin cDNA hybridised to Eco R1 fragments derived from the A_1 and A_2 genes but not to fragments from the B_1 and B_2 genes. These conditions therefore permit hybridisation between 5% mismatched sequences and prohibit hybridisation between 20% mismatched sequences. Thus, both estimates suggest that the degree of homology between MuMTV and the related T-47D DNA sequences is >80%.

Subgenomic Pst 1 fragments of GR-MuMTV (28,29 ; see Fig. 1) cloned in pBR 322 were hybridised to Eco R1 digested T-47D DNA. This approach was chosen to increase the sensitivity of the assay and to map the sequences related to human DNA within the viral genome. The long terminal repeat is contained in the 1.45 kb fragment (LTR probe), the 5′ end of the gag gene which encodes the viral core proteins in the 1.1 kb fragment (gag probe) the remainder of the gag gene and the pol gene coding for the reverse transcriptase in the 4.0 kb fragment which spans the internal Eco R1 site (gag-pol probe) and the env gene coding for the viral envelope proteins, gp 52 and gp 36, in the 2.0 kb fragment (env probe).

The autoradiograms in Fig. 3 show that in 20% formamide all 4 of the MuMTV subgenomic fragments hybridise to discrete

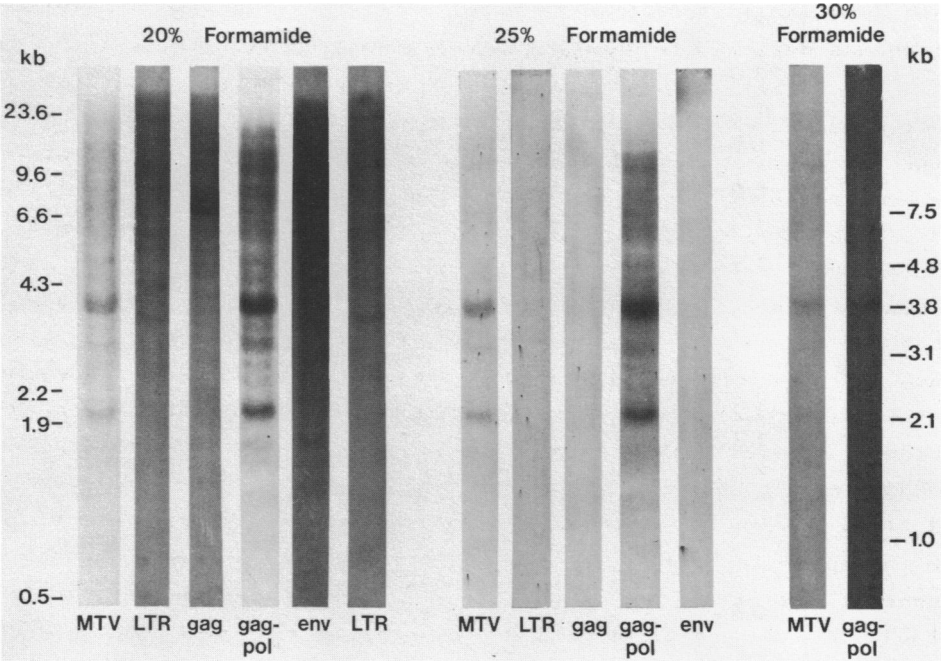

Figure 3. Hybridisation of subgenomic MuMTV fragments to T-47D cell DNA.

Eco R1 digested T-47D cell DNA was electrophoresed, transferred to nitrocellulose and hybridised in solutions containing 20%, 25% or 30% formamide, as described in the Materials and Methods. The hybridisation probe used (see Fig. 1) is indicated beneath each of the tracks which are aligned in the relative order of the probes in a MuMTV provirus ; thus the hybridisation with the LTR probe is shown adjacent to hybridisation with both the gag probe and the env probe. The autoradiograms of the filters hybridised in 20% formamide to the LTR, gag and env probes were exposed 3 times as long as those of the filters hybridised to the MuMTV and gag-pol probes. The positions of the molecular weight markers are indicated on the left and those of the T-47D Eco R1 fragments detected by MuMTV in the experiment shown in Fig. 2 on the right.

Eco R1 fragments in T-47D DNA. In general, the LTR, gag and env probes gave weak hybridisation to fragments which were not detected using total MuMTV DNA as the hybridisation probe. The gag-pol probe gave the strongest hybridisation and the profile, apart from some additional fragments, was very similar to that obtained with the entire MuMTV probe. Two linked MuMTV fragments

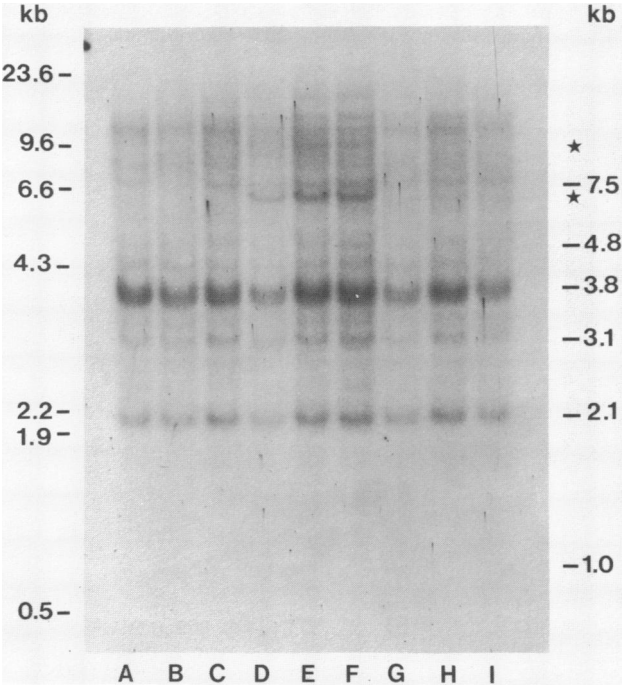

Figure 4. Hybridisation of the gag-pol probe to DNA from different human breast cancer cell lines and from normal human DNA.

Eco R1 digested DNA was prepared, electrophoresed and transferred to nitrocellulose. The filter was hybridised to nick-translated MuMTV DNA in the presence of 25% formamide, washed and exposed to film as described in the Materials and Methods. Hybridisation is to DNA from : wild-type T-47D cells (16), (A) ; T-47D clone 8 cells (17) (B) ; T-47D clone 11 cells (17) (C) ; MCF-7 subline a. cells, (D) ; MCF-7 subline b. cells (18), (E) ; R27 cells (37), (F) ; ZR 75 1 cells (38), (G) ; HBL 100 cells, (H) and human placenta, (I). The asterisks indicate the positions of the MuMTV-related Eco R1 fragments only present in DNA from sublines of MCF-7 cells. On the left, the positions of the molecular weight markers are indicated and on the right, those of the Eco R1 fragments detected by MuMTV in T-47D DNA (Fig. 2).

sometimes hybridised to Eco R1 fragments of the same size and this may be indicative of a similar linkage for the MuMTV-related sequences in human DNA. Only the gag-pol and MuMTV probes detected related sequences in human DNA after hybridisation in the presence of 25% formamide (Fig. 3) and, because the background was lower, their hybridisation was much clearer

than in 20% formamide. These probes still hybridised to the T-47D Eco R1 fragments of 2.1, 3.0 and ≃ 3.8 kb in the presence of 30% formamide (Fig. 3).

The MuMTV-related sequences in T-47D DNA could be inherited through the germline and therefore present in the DNA of all cell types or the result of an infection and subsequent integration of proviral DNA. MuMTV DNA was therefore hybridised to Eco R1 digested human DNA prepared from various breast tumour cell lines, a cell line derived from normal breast epithelial cells and human placenta (Fig. 4). The same MuMTV-related Eco R1 fragments as are present in the wild type T-47D cell DNA studied in this report (A) were detected in DNA from two cloned sublines of these cells (B and C), in DNA from another human breast cancer cell line, ZR 75 1 (G), in DNA from the cell line derived from normal breast epithelial cells, HBL 100 (H), and in human placental DNA (I). The DNAs of two sublines of a third human breast cancer cell line, MCF_7, and of a cloned tamoxifen-resistant subline all contained these fragments and two additional MuMTV-related Eco R1 fragments of 6.6 and 9.5 kb (D, E and F).

DISCUSSION

This study demonstrates the presence of MuMTV-related sequences in human DNA, some of which are more than 80% homologous with regions of the MuMTV genome. All four subgenomic probes, which together cover the entire MuMTV genome, hybridised to discrete Eco R1 fragments in human DNA. Hybrids formed by the gag-pol probe were the most stable which suggests that the gag-pol region of MuMTV shares the greatest homology with the human MuMTV-related sequences. Hybridisation with the gag, env and LTR probes required a somewhat lower stringency. The low homology between the env probe and human DNA may be somewhat surprising in view of the reported cross-reactivity between antigens in human breast tumours and antibodies raised against gp 52 of MuMTV (15). High degrees of homology, however, may be detected between proteins whose DNAs do not crosshybridise (39).

While this manuscript was in preparation, a study was published (40) which described the presence of human MuMTV-

related sequences and the isolation of a clone containing some of these sequences. The hybridisation obtained with their probes to Eco R1 digested human DNA is consistant with that reported in this study for the MuMTV, gag-pol and env probes. However, hybridisation of an LTR probe to human DNA was not reported and the authors could not detect LTR-related sequences in their cloned fragment of human DNA, which did contain sequences homologous to the gag-pol and env regions of MuMTV. The demonstration that MuMTV-LTR-related sequences are present in human DNA is clearly important ; considering that the control sequences for initiation and termination of retroviral transcription and the steroid hormone-receptor binding site are all contained within the LTR (41,4,5). Further, LTR insertion adjacent to and activation of a cellular oncogene is known to induce tumourogenesis (42,43) and has recently been implicated in mouse mammary carcinogenesis (44).

Although murine leukemia virus (MuLV) DNA does not hybridise to discrete fragments of human DNA (45), even under the conditions of lowered stringency used in this study (data not shown), two retroviral-like sequences with homology to MuLV DNA have recently been isolated from human DNA (46,47). The first has some homology with the 3' end of the gag gene and more with the pol gene of MuLV (46). Sequence comparison of the second with MuLV demonstrated 57% conservation overall and up to 68% in the pol genes, some in the gag gene and LTR but no detectable homology between env genes (47). Thus, although MuLV appears to be less related to its human counterpart than MuMTV is related to the human sequences described in this study, the relative homologies between different viral legions and their related human sequences are similar.

The presence of MuMTV-related sequences in placental DNA suggests that at least some of the fragments found in breast tumour DNA are present in normal human DNA. MCF_7 cells do however contain MuMTV-related sequences which are not present in placental DNA. This finding is in contrast to those reported by Callahan et al (40) who only identified the same fragments in MCF_7 DNA as they found in normal DNA. These results also show that the human MuMTV-related sequences may differ between cells

derived from different tumours. These differences could reflect genetic polymorphism or the new insertion of MuMTV-related sequences in the MCF_7 genome. The full significance of these differences and the structure, expression and regulation of these sequences is under analysis.

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*To whom reprint requests should be addressed.

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