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Molecularly cloned c-*mos*(rat) is biologically active

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A unique rat cellular gene, c-*mos*(rat), homologous to the transforming sequences, v-*mos*, of Moloney murine sarcoma virus (M-MSV) was detected by hybridization to a v-*mos* specific probe. The c-*mos*(rat) gene was cloned together with its flanking sequences in an 11-kbp *Eco*RI DNA fragment inserted in vector Charon 4A. Two probes were used to investigate the position and orientation of c-*mos*(rat) in the clone examined (D_3e), namely pMSV-31 which contains the sequences specific for the transforming sequences of M-MSV and pCS-1 which harbors 0.5 kbp of 5'-terminal sequences of c-*mos*(mouse) as well as 0.7 kbp of its flanking sequences. After ligation of a restriction fragment of clone D_3e containing c-*mos*(rat) to a fragment containing the long terminal repeat of M-MSV and transfection of the DNA onto rat cells, we detected foci of transformed cells, thus showing that c-*mos*(rat) is biologically active. Using DNA framents derived from clone D_3e, we studied the conservation of c-*mos* and of its flanking sequences in several species. c-*mos*(rat) as well as some of its flanking sequences appeared to be highly conserved in the species studied.

Key words: conserved sequences/oncogenes/transfection

Introduction

Moloney murine sarcoma virus (M-MSV) causes rapidly developing sarcomas in mice as well as malignant transformation of cultured fibroblasts (Moloney, 1966). The viral gene responsible for the oncogenic properties is called v-*mos*, (v from viral). The virus acquired this gene, and indeed its very identity, by a recombination between Moloney murine leukemia virus (M-MLV) and mouse cellular sequences, designated c-*mos* (c from cellular) (Van Beveren *et al.*, 1981a). The *mos* gene belongs to a group of originally cellular genes, collectively called oncogenes, that through recombination events gave rise to new transforming type C RNA tumor viruses (Coffin *et al.*, 1981). Much research is focussed on cellular homologues of these genes, because it is believed that their abnormal expression may be involved in non-viral tumorigenesis; recent reports describe the expression of some c-*onc* genes in human tumors (Goldfarb *et al.*, 1982; Der *et al.*, 1982; Eva *et al.*, 1982). In addition to potentially malignant properties, the c-*onc* sequences are believed to fulfill as yet unknown but important biological functions, possibly in development and differentiation of the organism (Eva *et al.*, 1982; Westin *et al.*, 1982). This speculation is mainly based on the slow evolutionary rate of these sequences. Homologues of c-*onc* genes are usually found in a wide variety of vertebrates (Stéhelin *et al.*, 1976; Frankel and Fischinger, 1977; Spector *et al.*, 1978; Sheiness and Bishop, 1979; Goff *et al.*, 1980; Shilo and Weinberg, 1981).

Unlike some other *onc* genes, the c-*mos* gene and its product are not very well characterized. Only very low levels of v-*mos* related protein have been detected in M-MSV transformed cells (Papkoff *et al.*, 1982). The product was characterized in *in vitro* translation studies (Papkoff *et al.*, 1981). In mouse (Jones *et al.*, 1980, Oskarsson *et al.*, 1980) and man (Watson *et al.*, 1982) cloned c-*mos* sequences have been described. The c-*mos*(mouse) exists in a hypermethylated form in mouse cells: no expression is observed (Gattoni *et al.*, 1982). In this paper we report data on c-*mos*(rat) and its transfection and apparent expression in rat cells. This study led also to the discovery of a unique and very conservative sequence that flanks c-*mos* of mouse and rat and is present in other vertebrates.

Results

Characterization of c-mos(rat)

To detect rat cellular sequences homologous to v-*mos* and to compare them to c-*mos*(mouse), we isolated high mol. wt. DNA from 3Y1 rat cells and NIH-3T3 cells. DNAs were digested with *Eco*RI, *Pst*I, or with a combination of *Eco*RI and *Hind*III, separated on an agarose gel, blotted and hybridized to nick-translated pMSV-31 (detecting v-*mos* homologous sequences) or pCS-1 (detecting 5'-terminal c-*mos* as well as adjacent mouse cellular sequences). The results are shown in Figure 1. From Figure 1A it can be seen that mouse DNA contains c-*mos* sequences hybridizing to pMSV-31 on one *Eco*RI DNA fragment of 14 kbp (lane d) and on a *Pst*I DNA fragment of 2.0 kbp (lane c). Several small *Pst*I DNA fragments hybridize to pMSV-31 (Jones *et al.*, 1980), but they are not detectable in this experiment. A rat *Eco*RI DNA fragment of 11 kbp hybridizes to pMSV-31 (lane b): some *Pst*I DNA fragments reactive with pMSV-31 as a probe were too weak to be seen in this experiment (lane a).

A further characterization was obtained using pCS-1 as a probe in a comparison between mouse and rat DNAs. This comparison is shown in Figure 1B. Lanes b and d show an *Eco*RI/*Hind*III digestion of mouse DNA: a single fragment of 3.9 kbp hybridizes to pCS-1 (lane b) and to pMSV-31 (lane d) as was expected from earlier restriction data (Jones *et al.*, 1980). However, *Eco*RI/*Hind*III digested rat DNA reveals one DNA fragment of 1.5 kbp hybridizing to both pCS-1 (lane a) and pMSV-31 (lane c) as well as an additional DNA fragment of 3.2 kbp hybridizing only to pCS-1 (lane a), indicated by the arrow.

Therefore, it appears that rat DNA contains sequences homologous to c-*mos*(mouse) as well as sequences homologous to mouse DNA juxtaposed to the c-*mos* gene. It will follow from the experiments described below that in the rat genome these two sequences are also very close.

Restriction mapping of cloned c-mos(rat)

The *Eco*RI DNA fragment, harboring c-*mos* related sequences (see Figure 1A, lane b), was cloned as described. Five

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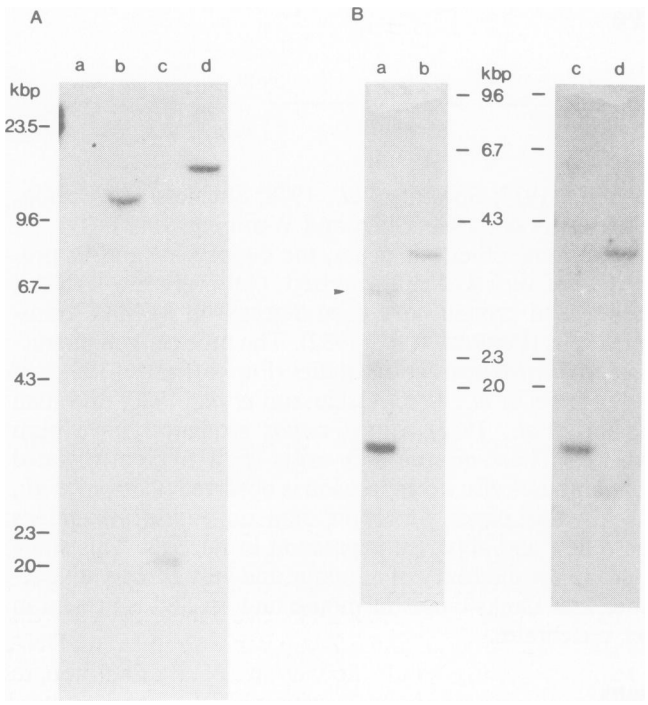

Fig. 1. Detection of c-*mos*(rat). **(A)** High mol. wt. DNAs from rat **(lanes a** and **b)** or from mouse **(lanes c** and **d)** were digested with *Pst*I **(lanes a** and **c)**, or with *Eco*RI **(lanes b** and **d)**. After electrophoretic separation in a 0.7% agarose gel and blotting onto a nitrocellulose filter the DNA fragments were detected by hybridization to pMSV-31. **(B)** In *Hind* III/*Eco*RI digested rat DNA **(lanes a** and **c)** or mouse DNA **(lanes b** and **c)** c-*mos* and flanking sequences were detected using pCS-1 **(lanes a** and **b)** or pMSV-31 **(lanes c** and **d)** as probes.

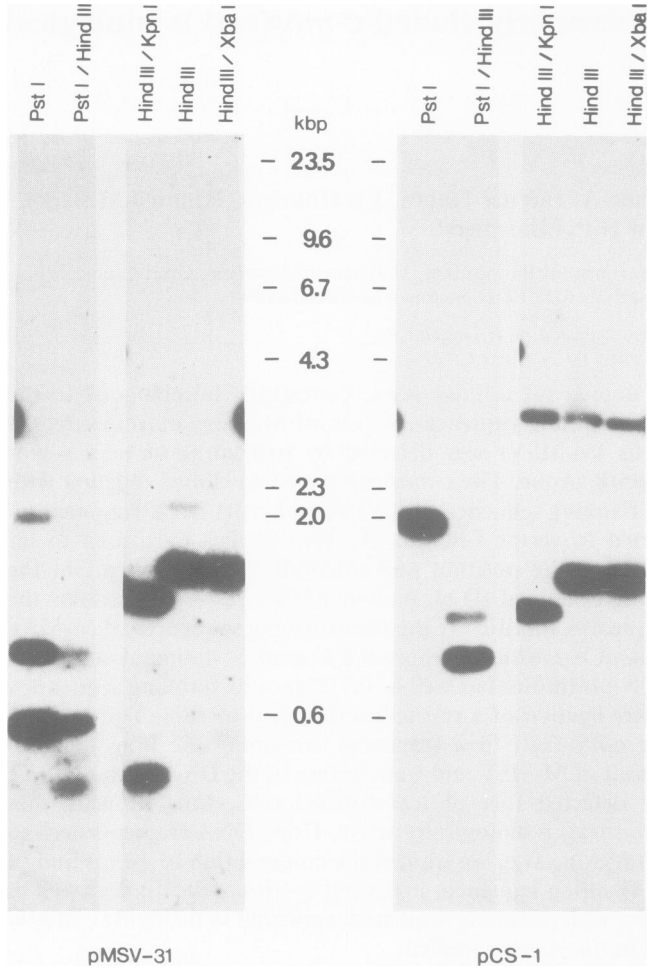

Fig. 2. Positioning of c-*mos*(rat) in clone D_3e. 2 ng of D_3e DNA was first digested with *Eco*RI and then with the enzymes indicated. Detection of DNA fragments was as described in the legend to Figure 1.

out of 15 positive clones were grown and digested with *Eco*RI and *Hind*III and analysed on agarose gels. They appeared to be indistinguishable by restriction analysis and to contain an 11-kbp *Eco*RI DNA insert. One of these clones, designated D_3e, was used for further analyses.

Using pMSV-31 and pCS-1 as probes we determined the position of c-*mos* on the 11-kbp DNA fragment. Figure 2 shows part of these restriction analyses. The left panel shows an autoradiograph of a blot containing D_3e restriction fragments hybridized to pMSV-31 and the right panel shows an autoradiograph of an identical blot hybridized to pCS-1. The additional DNA fragments showing up on the right panel result from hybridization of rat DNA sequences to the mouse DNA sequences flanking the c-*mos*(mouse). Although the environment of c-*mos* in mouse and rat is not completely identical (Figure 1) this experiment shows that sequences in the neighborhood of both genes are homologous. *Hind*III digestion results in DNA fragments of 1.5 and 3.2 kbp. The latter fragment is only detectable using pCS-1 as a probe as was expected from the results shown in Figure 1B. This DNA fragment is cleaved by *Xba*I, resulting in a slightly smaller DNA fragment of 3.1 kbp (Figure 2, right panel). *Kpn*I cleaves the c-*mos* sequences once: the small *Kpn*I/*Hind*III DNA fragment of 450 bp can only be detected using pMSV-31 as a probe. In this figure, *Pst*I DNA fragments are observed containing c-*mos*(rat) sequences and flanking sequences that were not detectable in the experiment shown in Figure 1. From these and other data, a restriction map of clone D_3e was obtained. In Figure 3 it is compared to the restriction map of c-*mos*(mouse) (Jones *et al.*, 1980; Oskarsson *et al.*, 1980). The regions in D_3e hybridizing to pMSV-31 and pCS-1 are also indicated.

Biological activity of c-mos(rat)

As was shown by Blair *et al.* (1981) the c-*mos*(mouse) gene has no transforming activity in transfection assays unless the gene is ligated to activating sequences like retroviral long terminal repeats (LTRs) (Temin, 1982). By digestion with *Sma*I we isolated LTR sequences from a plasmid pMLTR that contains two M-MSV LTRs in the same orientation. Nucleic acid sequence data showed the presence of a single *Sma*I site in the 3′ region of this LTR (Van Beveren *et al.*, 1981a). The resulting 0.6-kbp DNA fragment containing the cyclically permuted LTR sequences was ligated to a *Sma*I DNA fragment harboring c-*mos*(rat) (Figure 4). The ligated DNA, as well as several other DNA fragments, was transfected onto rat cells and transformed colonies were counted after 2–3 weeks. Figure 4 shows the results of the transfection experiments. It can be seen that the 11-kbp *Eco*RI insert and the LTR sequences are not biologically active by themselves. Also, removal of large parts of the adjacent rat cellular sequences by digestion with *Hind*III or *Xba*I, does not result in detectable activity. However, the DNA preparation consisting of c-*mos*(rat) ligated to LTR sequences gives rise to transformation of cells in tissue culture after transfection. Therefore, the clone D_3e contains a functional c-*mos*(rat) gene that can be activated by ligation to enhancing LTR sequences.

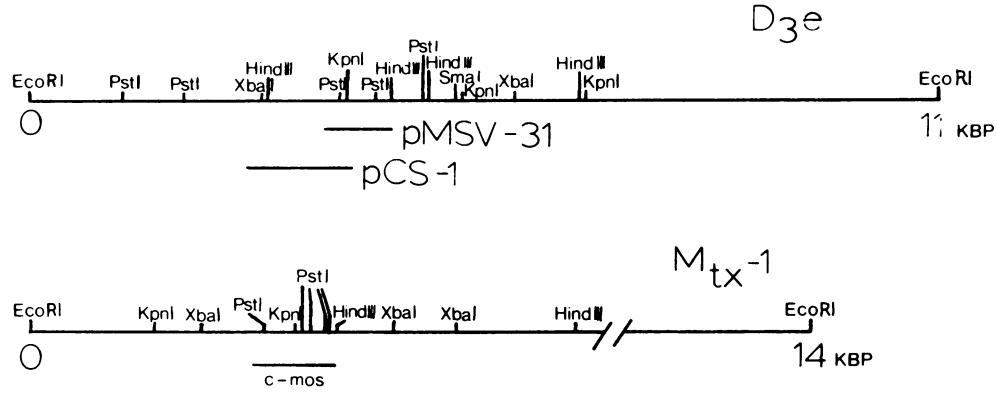

Fig. 3. Restriction map of D_3e. The restriction map of clone D_3e containing c-*mos*(rat) is compared to the restriction map of clone M_{tx}-1 (Jones *et al.*, 1980) containing c-*mos*(mouse). Fragments hybridizing to pMSV-31 or to pCS-1 are underlined.

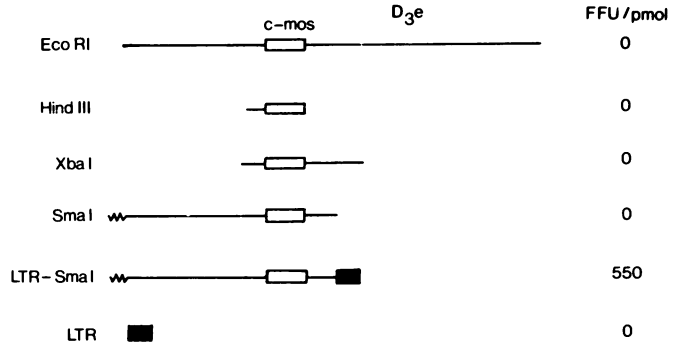

Fig. 4. Biological activity of c-*mos*(rat). The insert of clone D_3e, fragments thereof, LTR sequences, or the D_3e fragment ligated to the LTR sequences were transfected onto rat cells and transformed colonies were counted after 2–3 weeks (see Materials and methods). FFU/pmol = focus forming units per pmol DNA.

Conservation of rat cellular sequences in other species

Several cellular analogs of viral transforming sequences have been detected in the DNA of other species than those from which the viruses were isolated originally (Stéhelin *et al.*, 1976; Ellis *et al.*, 1980; Goff *et al.*, 1980; DeFeo *et al.*, 1981). Thus, the clone D_3e could be used to investigate the presence of cellular sequences homologous to c-*mos*(rat) in other species. Moreover, the fact that mouse DNA present in pCS-1, other than c-*mos*, hybridized to a unique fragment in rat DNA present in clone D_3e suggested that this DNA sequence juxtaposed to c-*mos* in mouse and rat might be conserved throughout several species.

Therefore, we isolated two restriction fragments designated P-H and H-H from clone D_3e, as indicated in Figure 5A. These fragments, as well as pMSV-31, were nick-translated and used as probes to screen filters containing separated *Eco*RI DNA fragments of hamster, mink, cat, pig, *Xenopus*, and pea DNA. From Figure 5B it can be seen that pMSV-31 recognizes sequences homologous to v-*mos* in various mammalian DNAs. Each of the DNAs from hamster, mink, cat, and pig contain one *Eco*RI DNA fragment hybridizing to pMSV-31 (of 15, 4.9, 9.6, and 5.4 kbp, respectively). In *Xenopus* and pea DNAs, of the latter we used two separate batches, no fragments were detected with pMSV-31 (data not shown). When using H-H as a probe we obtained the same results (data not shown).

We then investigated whether or not similar DNA fragments could be detected with probe P-H. Figure 5C shows the results obtained with the nick-translated P-H probe. In rat DNA three bands are detected, namely the 11-kbp DNA fragment containing c-*mos*(rat), a 9.5- and a 6.7-kbp fragment. In hamster DNA two bands are visible that comigrate with the 9.5- and 6.7-kbp bands found in rat DNA. In mink DNA three bands show up: no band of 4.9 kbp, the size of the fragment detected using pMSV-31 as a probe, is visible. In *Eco*RI-digested cat DNA one band of 8.0 kbp, in *Eco*RI-digested pig DNA a band of 9.6 kbp, and in *Eco*RI-digested *Xenopus* DNA one band of 5.0 kbp is visible. A strongly hybridizing 3.9-kbp *Eco*RI DNA fragment is observed in pea DNA, although we could not detect fragments in pea DNA using pMSV-31 as a probe. The data shown indicate that sequences homologous to c-*mos* and to its juxtaposing sequences are present in low copy numbers in closely or more distantly related species. All species examined contained DNA fragments homologous to rat cellular sequences represented in probe P-H, but only in mouse and rat do these sequences lie next to c-*mos* on one *Eco*RI DNA fragment.

Discussion

We have shown that sequences homologous to M-MSV-specific sequences (v-*mos*) are present in normal rat DNA on one unique 11-kbp *Eco*RI fragment. These sequences can be activated to transform cells *in vitro* upon transfection. Recently, Watson *et al.* (1982) reported that the human c-*mos* gene could not be activated in similar transfection experiments. The detection of a long open reading frame made it unlikely that a mutation caused the absence of transforming activity. Rather, it might be that c-*mos*(human) has diverged from c-*mos*(mouse) and the closely related c-*mos*(rat), thereby losing the capacity to transform cells. The position of c-*mos* on clone D_3e was determined using pMSV-31 as a probe and the orientation was determined using pCS-1 as a probe. The orientation of the strand homologous to M-MSV RNA in clone D_3e is from the left to the right in Figure 3.

Type C retroviral LTRs or parts thereof have been used successfully to activate genes (Blair *et al.*, 1981; DeFeo *et al.*, 1981; Temin, 1982). Nucleic acid sequence data have shown that they harbor a termination signal, a poly(A) addition site, and a promoter region. In addition, sometimes they contain repeated sequences of variable length that can be used as enhancer sequences in experiments designed to activate genes of interest (Levinson *et al.*, 1982). In similar experiments, we used the permuted M-MSV LTR, obtained by cleaving

pMLTR with *Sma*I. The *Sma*I site is located to the right of the promoter sequences in this LTR (Van Beveren *et al.*, 1981b). The ligated DNA preparations, used for transfections, appeared to consist for the major part of the product shown in Figure 4, as well as of DNA consisting of concatamers containing several LTRs (not shown). The ligation step was necessary for efficient transfection to occur. The transfection efficiency we observed was much lower than the efficiency reported for c-*mos*(mouse) (Blair *et al.*, 1981).

Using a cDNA specific for the transforming gene *(src)* of Rous sarcoma virus, a chicken virus, Stéhelin *et al.* (1976) found that the degree of homology between the *src* gene and its cellular homologues decreased with increasing evolutionary distance between the species investigated and the chicken. Cellular homologues of transforming genes of RNA tumor viruses are present in many different animal species (Coffin *et al.*, 1981). In our assay, using specific probes (Figure 5A), we found that the degree of homology between v-*mos* and c-*mos* was highest for mouse and rat and less for mink, hamster, cat, and pig. Interestingly, probe P-H, representative for sequences juxtaposed to c-*mos* both in mouse and rat, detected homologous sequences in low copy number in all species examined, including a plant. However, only in the case of rat and mouse is the same *Eco*RI fragment detected using probes P-H and pMSV-31. The detection of different fragments with these probes for hamster, mink, cat, and pig could be caused by the presence of an additional *Eco*RI site or by a different organization of the sequences analysed. The 3.9-kbp fragment detected in pea DNA, under rather stringent hybridization conditions, might, however, be caused by a fragment present as a repetitive sequence with some homology to probe P-H. We do not know the relevance of this sequence, but the close association of these unique sequences to c-*mos* in rat and mouse and the conservation throughout other species, suggest that they fulfill some important function.

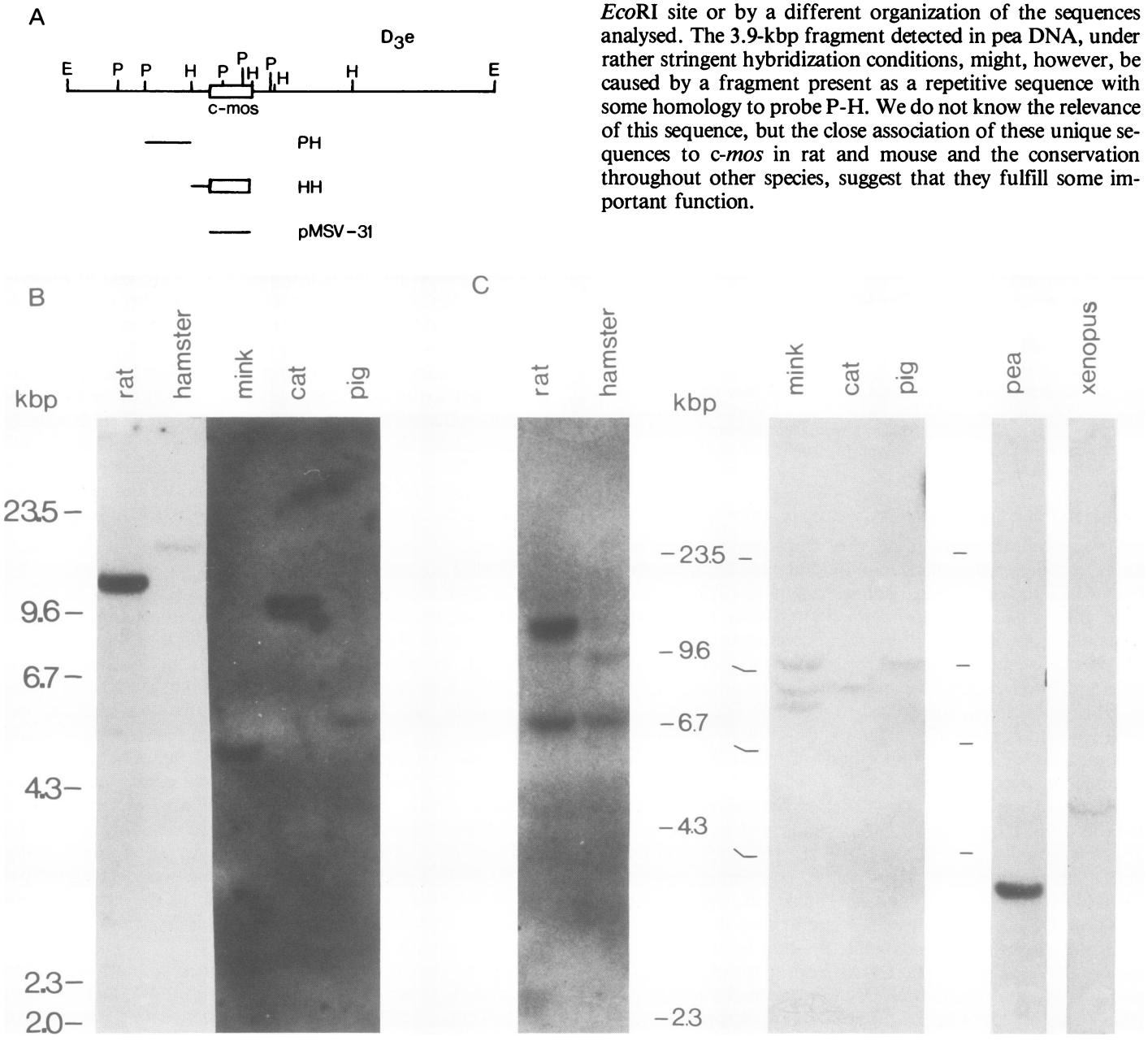

Fig. 5. Conservation of c-*mos* and its flanking sequences. **(A)** Map of the fragments used as probes for detection of c-*mos* related sequences (pMSV-31) or its flanking sequences (fragment P-H). E = *Eco*RI, P = *Pst*I, and H = *Hind*III. **(B)** Autoradiograph of a nitrocellulose filter containing *Eco*RI DNA fragments of the species indicated, hybridized to nick-translated pMSV-31. **(C)** Autoradiograph of a nitrocellulose filter containing *Eco*RI DNA fragments of the species indicated, hybridized to nick-translated probe P-H. In **(B)** and **(C)** the exposure times were 1 day for the lanes marked 'rat' and 'hamster', and 10 days for the other lanes.

Materials and methods

Cells and transfection procedure

The cell lines 3Y1 (rat), Rat-2 (rat), NIH-3T3 (mouse), Balb/c-3T3 (mouse), CCL-64 (mink), PK-15 (pig), AZ (cat), and Halec (hamster) were grown on Dulbecco's modified Eagle's medium, supplemented with 10% calf serum (GIBCO) and antibiotics. Transfection was performed essentially as described by Graham and Van der Eb (1973). In short: Rat-2 cells were plated 4 h before transfection in Petri dishes (Costar, 35 mm). 20 min after precipitation of cloned DNA fragments with $CaCl_2$ the DNA precipitate was applied to the cells and incubated for 5 h. After removal of the medium the cultures were incubated for 1 h after which they were transferred to 100 mm Petri dishes (Costar). Cells were fed medium every 3 days and foci were counted after 2–3 weeks.

Cloning procedure

Rat cellular DNA was isolated from 3Y1 cells as described (Van der Putten *et al.*, 1979), and 100 μg DNA was digested with *Eco*RI (Boehringer) and separated on a 0.7% agarose gel (SeaKem). The gel was sliced and DNA was electro-eluted (Allington *et al.*, 1978). Fractions containing sequences homologous to v-*mos* were detected after blotting on nitrocellulose filters by the Southern technique (Southern, 1975) by hybridization to pMSV-31. Hybridizations were performed at 42°C for 15 h in 50% formamide, 5 x SSC, 1 x Denhardt's solution, 0.02 M sodium phosphate, 100 μg/ml denatured herring sperm DNA and 0.5–1 x 10^6 c.p.m./ml of probe DNA. The final wash was for 15 min at 62°C in 0.1 x SSC/0.1% SDS (Jones *et al.*, 1980). An 11-kbp fragment containing v-*mos* homologous sequences was cloned in Charon 4A as described (Jones *et al.*, 1980). From 100 000 plaques screened we obtained 15 plaques harboring phages with v-*mos* homologous sequences. Five of these were grown and appeared to contain the same insert. One of these, D_3e, was used for characterization.

Clones used

pMSV-31: pBR322 containing the v-*mos* sequences as an insert in the *Pst*I site (Jones *et al.*, 1980). pCS-1: pBR322 containing 0.5 kbp of 5′-terminal c-*mos*(mouse) sequences as well as 0.7 kbp of its flanking sequences (Van Beveren *et al.*, 1981b). pMLTR: pBR322 containing two adjacent LTRs and flanking M-MSV sequences, inserted in the *Eco*RI and *Pst*I sites of the plasmid.

Restriction enzyme analysis and ligation of DNA fragments

DNAs were digested with *Eco*RI (Boehringer), *Kpn*I, *Pst*I, *Xba*I, *Bam*HI, *Sma*I, or *Hind*III (Bethesda Research Laboratories) as prescribed by the manufacturers. After separation on 0.7% agarose gels (SeaKem) the DNA fragments were processed as described under 'cloning'. DNA fragments to be ligated were isolated from gel slices by electro-elution, ethanol precipitated twice and ligated using T4 DNA ligase (Boehringer).

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