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Localization of the *mcf*.2 transforming sequence to the X chromosome

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A transforming sequence was identified using co-transfection of DNA from the human mammary carcinoma cell line MCF-7 and of a G418 resistance gene into NIH 3T3 cells, followed by tumor formation in athymic mice. This sequence, named *mcf*.2, was molecularly cloned. A transforming activity resides in a cosmid clone of 42 kb. *mcf*.2 did not cross-hybridize with the known oncogenes tested. *In situ* hybridization localized it on the X chromosome, probably at q27. This localization was confirmed by hybridization to a panel of human-rodent cell line DNAs.

Key words: molecular cloning/*in situ* hybridization/transformation/tumorigenicity

Introduction

The presence of activated cellular genes in human neoplasms was detected by focus formation in murine fibroblasts following transfection with DNA extracted from various tumors or cell lines. In all but a few cases (Cooper *et al.*, 1984; Eva and Aaronson, 1985; Martin-Zanca *et al.*, 1986; Padua *et al.*, 1984; Takahashi *et al.*, 1985), the oncogenes thus identified were members of the *ras* family (Reddy *et al.*, 1982; Shimizu *et al.*, 1983a, b; Tabin *et al.*, 1982). Putative transforming genes were also evidenced using alternative assays (Padhy *et al.*, 1982; Cochran *et al.*, 1984; Fasano *et al.*, 1984; Müller and Müller, 1984). One such assay, based on the formation of tumors in nude mice after inoculation of NIH 3T3 cells co-transfected with neoplastic DNA and a G418 antibiotic resistance gene, has been described previously (Fasano *et al.*, 1984). Using this assay, three transforming genes, called respectively *mcf*.1, *mcf*.2 and *mcf*.3, were initially isolated from nude mouse tumors obtained upon transfection of NIH 3T3 cells with DNA from the human mammary carcinoma cell line MCF-7. Further characterization of these putative oncogenes showed that *mcf*.1 was a N-*ras* gene and that *mcf*.3 arose by recombination of a normal human c-*ros* gene during transfection (Birchmeier *et al.*, 1986). We report here the molecular cloning and chromosomal localization of the *mcf*.2 transforming locus.

Results

Molecular cloning of the mcf.2 locus

Results of experiments of co-transfection with DNA from the human mammary carcinoma cell line MCF-7 have been described previously (Fasano *et al.*, 1984). In brief, DNA isolated from MCF-7 cells was co-transfected into NIH 3T3 cells with the pKOneo plasmid carrying the G418 antibiotic resistance gene. Cells resistant to the antibiotic were grown into colonies, pooled and injected into nude mice. The MCF-7-2 tumor developed within 5 weeks. The same procedure was repeated using DNA from this primary tumor. Cell lines were derived from the secondary tumors thus obtained; hybridization with total nick-translated human DNA revealed that they all contained a common human gene, which was called *mcf*.2 (Fasano *et al.*, 1984).

A set of lambda phage clones was isolated previously from one of the secondary tumors, MCF-7-2-2; this initial cloning took advantage of the presence of Alu repeat sequences in the transferred human DNA (Fasano *et al.*, 1984). Further analysis revealed that these recombinant phages, later shown to be contained within the G2 and H4 cosmid clones (Figure 1A) spanning >30 kb of DNA, were only part of the *mcf*.2 locus; they exhibited no transforming activity in an *in vitro* assay. A cosmid library was then constructed, by inserting sized (35-45 kb) *Eco*RI fragments from MCF-7-2-2 DNA into the pHC79 cosmid vector. An appropriate *Eco*RI fragment of 2.4 kb (Figure 1B) was purified from one of the phage clones, and used as a probe to isolate cosmid clones containing *mcf*.2 sequences. A mixture of three *Eco*RI fragments of 3.4, 3.3 and 3.2 kb, purified from cos A6, was subsequently used in a second round of screening. Representative cosmid clones are shown in Figure 1A. From the overlapping cosmid clones, an *Eco*RI map, encompassing >100 kb of cloned human DNA, was deduced (Figure 1B). On the left side of the map, the presence of mouse repetitive sequences indicated the region of recombination of the transfected human DNA with the mouse genome (Figure 1B, dotted boxes). The various cosmid clones were also digested with *Eco*RI, and the blots thus obtained were hybridized with total nick-translated human DNA, which revealed the presence of human repetitive sequences in most portions of *mcf*.2 (Figure 1B, dashed boxes).

A cDNA library was constructed by cloning the cDNAs synthesized from poly(A)$^+$ RNA purified from MCF-7-2 cells into the λgt 10 cloning vector (Huynh *et al.*, 1984). Several cDNA clones were obtained, using as probes different *Eco*RI fragments isolated from the cosmid clones. Hybridization of the cDNA clones to *Eco*RI-digested cosmid clones localized transcribed sequences to the three *Eco*RI fragments of 3.3, 4.5 and 6.2 kb, respectively, present in the cosmid M2 (cos M2) (Figure 1A and B).

In addition, a cosmid library, prepared from MCF-7 DNA as described above, was screened with the 4.5-kb fragment of cos M2. Two independent overlapping clones were obtained (Figure 1D); their *Eco*RI restriction map was compared with that defined by the tumoral clones (Figure 1B). Identity of the two maps was observed in the region of the *mcf*.2 locus corresponding to part of cos M2 (Figure 1E), but they diverged ~6 kb upstream from cos M2. For the time being, we have been unable to clone, from MCF-7 DNA, the portion corresponding to the right part of cos M2.

We also attempted to clone the *mcf*.2 region corresponding to transcribed sequences from a non-tumoral DNA source. A

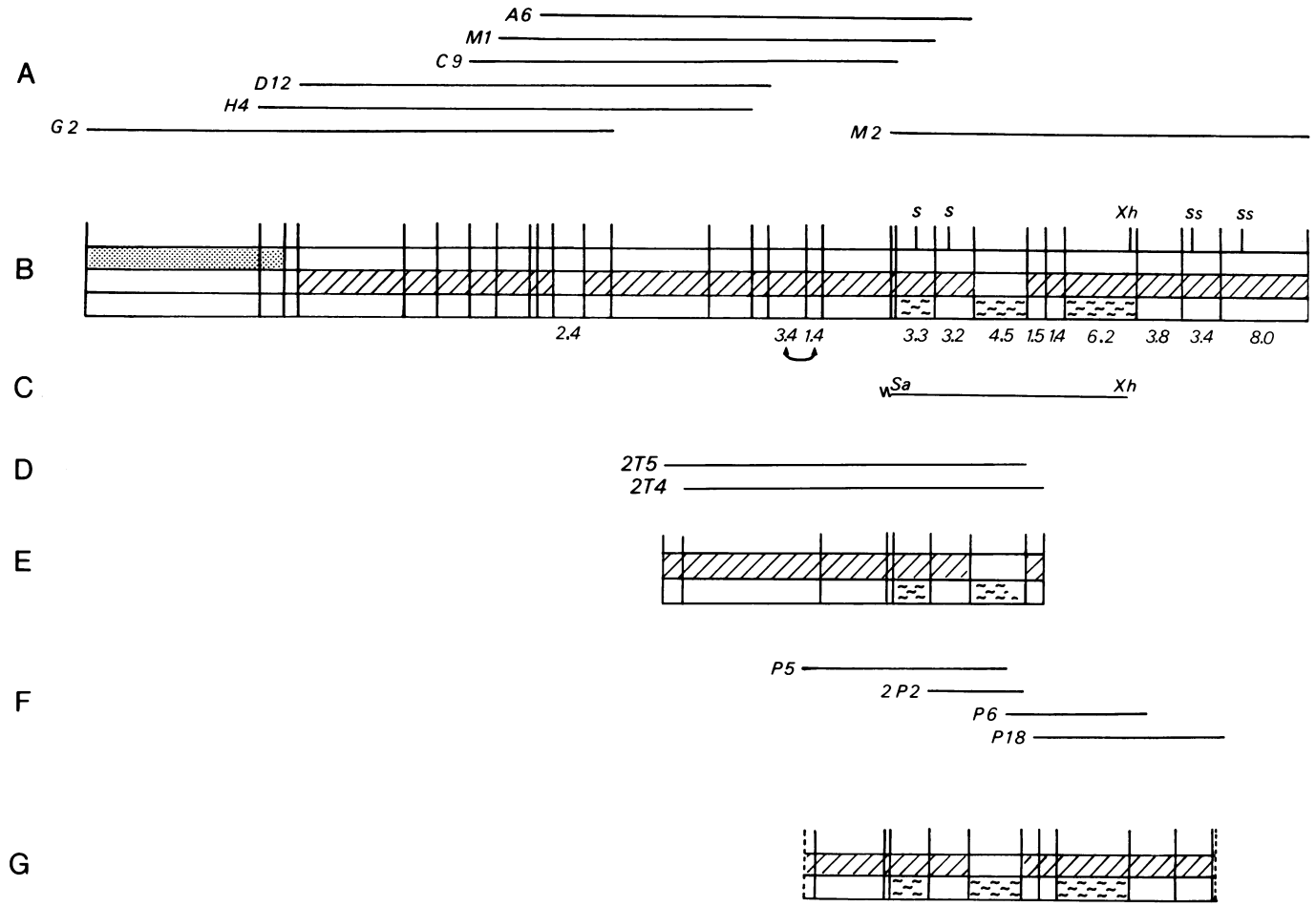

Fig. 1. Organization of the *mcf*.2 locus. **(A)** *Eco*RI inserts of independent cosmid clones derived from the MCF-7-2-2 tumor. The names of the various cosmids (for example cos A6) are indicated. **(B)** Composite *Eco*RI restriction endonuclease map of the cosmid clones. Vertical lines indicate *Eco*RI sites. The relative positions of the *Eco*RI fragments were determined from digests of cosmids containing overlapping inserts. Numbers below the fragments are their lengths in kb. The ambiguity in the relative positions of two fragments is indicated by the arrow. Abbreviations for other enzymes are: S: *Sma*I, Ss: *Sst*I and Xh, *Xho*I. These sites are indicated only for cos M2. For each fragment, boxes indicate hybridization signal with total mouse DNA (upper boxes, dotted), total human DNA (middle boxes, slashed), and *mcf*.2 cDNA clones (lower boxes, waved). **(C)** The *Sal*I–*Xho*I fragment of 20 kb, and containing a transforming activity, is indicated (Sa: *Sal*I and Xh: *Xho*I sites). **(D)** *Eco*RI inserts of independent cosmid clones derived from MCF-7 cells. **(E)** Composite *Eco*RI restriction map of the cosmid clones from MCF-7 cells. Symbols for the boxes are the same as in **B**. **(F)** Inserts of λ phage clones homologous to *mcf*.2 and derived from a library of normal peripheral blood lymphocytes, as well as a cosmid clone (2P2) derived from a library of placental DNA. **(G)** Composite *Eco*RI restriction map of the non-tumoral *mcf*.2 gene. Symbols for the boxes are the same as in **B**. Vertical dotted lines indicate the external *Sau*3A sites used for λ cloning.

lambda phage library, constructed with DNA extracted from peripheral blood lymphocytes, was screened. Three λ clones, P5, P6, and P18, reconstituted the *mcf*.2 region (Figure 1D). The reconstructed *Eco*RI map of these three recombinant phages (Figure 1E), was identical to the map of the corresponding *mcf*.2 region from MCF-7-2-2. Phages P5 and P6 did not overlap. In addition, a cosmid clone, 2P2, isolated from a human placental DNA library, and containing a small insert, overlapped both the P5 and P6 clones (Figure 1D).

Transforming capacity of mcf.2 clones

In order to determine if the molecular clones of *mcf*.2 thus isolated exhibited a transforming activity, the various cosmid clones were tested for their ability to transform NIH 3T3 cells upon transfection. Two assays were used: the induction of morphologically transformed foci, and the induction of tumors in nude mice. Results of these experiments are shown in Table I. Cos M2 was able to induce the formation of foci in NIH 3T3 cells (Table IA). The efficiency of focus formation was low when compared with a cloned activated human Harvey *ras* gene (Taparowsky *et al.*, 1982). Cos M2-transformed cells were morphologically distinct from c-Ha-*ras*-transformed cells but did not appear very different from normal NIH 3T3 cells (data not shown). They were tumorigenic in nude mice. A representative clone of cos M2-transformed cells was called 3T3 mcf.2.1; its DNA was able to confer the tumorigenic phenotype to co-transfected NIH 3T3 cells (Table IB). Two other cosmid clones (cos A6 and cos G2), representing different portions of the *mcf*.2 locus, were neither able to induce foci in the focus assay (Table IA) nor to induce tumors in the tumorigenicity assay (Table IB). To localize more precisely the transforming portion of the *mcf*.2 locus, a restriction enzyme map of cos M2 was established, using *Sal*I, *Sma*I, *Sst*I and *Xho*I. *Sal*I only cleaved cos M2 in the pHC79 vector. As shown in Figure 1B, cos M2 was cleaved twice by *Sma*I and *Sst*I, respectively, and once by *Xho*I. Cos M2 DNA was therefore cleaved by these enzymes, and tested in a tumorigenicity assay. Cos M2 cleaved either by *Sal*I, or by *Sst*I or by *Xho*I was still able to confer the tumorigenic phenotype to co-transfected NIH 3T3 cells, whereas *Sma*I-cleaved cos M2 no longer showed this

Table I. Transforming and tumorigenic potentials of *mcf*.2 clones

A. Focus assay

Cloned DNA[a]	Efficiency of primary transfection (f.f.u./μg)	Tumorigenicity of the transfectants[b]
Cos A6	0	–
Cos G2	0	–
Cos M2	10	6/6
pT24	2×10^4	3/3

B. Tumorigenicity assay

Donor DNA[c]	Tumorigenicity of co-transfected NIH 3T3 cells[d]
3T3 mcf.2.1	4/4[e]
Cos A6 + NIH 3T3	0/4
Cos G2 + NIH 3T3	0/4
Cos A6 + cos G2 + NIH 3T3	0/4
Cos M2	1/2
Cos M2 + NIH 3T3	4/4[e]
*Sal*I-cleaved cos M2 + NIH 3T3	3/3
*Sma*I-cleaved cos M2 + NIH 3T3	0/4
*Sst*I-cleaved cos M2 + NIH 3T3	4/4
*Xho*I-cleaved cos M2 + NIH 3T3	4/4
*Sal*I–*Xho*I fragment of cos M2 + NIH 3T3	4/4

[a]The cosmid clones were described in Figure 1. pT24 is a cloned activated human H-*ras* gene (Taparowsky *et al.*, 1982). 2.5 μg of cloned cosmid DNA were transfected into NIH 3T3 cells as described under Materials and methods.
[b]Cell lines were derived from the transfection experiments and named 3T3 mcf.2.1 to −6. 2×10^6 transformed cells were inoculated to each nude mouse. Tumorigenicity is expressed as the ratio of mice with tumors versus the number of animals that were inoculated.
[c]Co-transfections were performed as described under Materials and methods, using 2.5 μg of intact cosmid DNA and 5 μg of cleaved cos M2 DNA. NIH 3T3 DNA (30 μg) was used as a carrier in most experiments.
[d]Co-transfected NIH 3T3 cells resistant to G418 were pooled, and 1×10^7 cells were inoculated to each mouse. Tumorigenicity is expressed as the ratio of mice with tumors versus the number of animals that were inoculated.Tumors appeared within 2–6 weeks. Animals were kept in experience for 12 weeks.
[e]Cell lines derived from the tumors were named 3T3 mcf.2.1-t1 to -t3, and cos M2-t1 to -t2, respectively.

capacity (Table IB). The *Sal*I–*Xho*I fragment from cos M2 was purified and tested in a tumorigenicity assay. This portion of the *mcf*.2 gene was able to induce tumors in nude mice.

DNAs from different cos M2-transfected cells and DNAs from different cell lines derived from nude mice tumors were digested with *Eco*RI, and the Southern blots thus obtained were hybridized with ^{32}P-labeled cos M2 DNA. *mcf*.2 transforming sequences were present in all the DNAs tested (Figure 2, lanes 2–4 and 7–9). The common fragments were 6.2, 4.5, 3.3, 3.2, 1.5 and 1.4 kb long, respectively; they were not observed in NIH 3T3 DNA (Figure 2, lane 5). The last two fragments of 1.5 and 1.4 kb, respectively, were only seen in lanes 3, 7 and 9 upon a longer exposure. Thus a transforming activity of the cloned *mcf*.2 locus was located between the *Sal*I and *Xho*I sites, encompassing six *Eco*RI fragments, three of which contained transcribed sequences. Examination of several transformed cell lines and tumors by Southern blotting showed the presence of variable numbers of copies of *mcf*.2; there was no striking difference in the morphologies of transformants with a relatively low copy number, like 3T3 mcf.2.3 or 3T3 mcf.2.1 (NIH 3T3 cells transfected with cos M2 in a focus assay), or transformants with a higher copy number of *mcf*.2, like MCF-7-2-2 or cos M2-t1 (a tumor cell line obtained by inoculation to nude mice of cos M2-transfected cells, see Table IB) (data not shown). In addition, the *mcf*.2 copy number appeared unchanged between the DNA from a transformed focus, such as 3T3 mcf.2.1, and that of a tumor, 3T3 mcf.2.1-t3, obtained in the tumorigenicity assay of 3T3 mcf.2.1 DNA (compare lanes 7 and 9 in Figure 2).

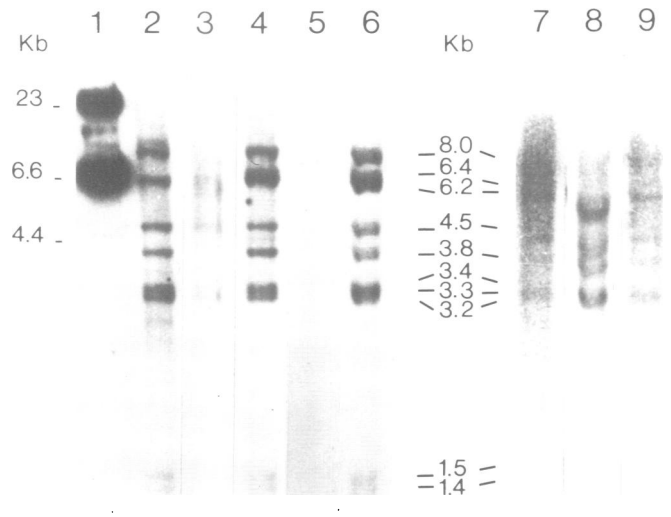

Fig. 2. Several nude mice tumors contain the same set of DNA fragments hybridizing with *mcf*.2. DNA preparations from several nude mice tumors and cos M2-transfected cell lines were digested with *Eco*RI and the blots hybridized with ^{32}P-labeled cos M2 DNA. The various lanes contained: *Hin*dIII fragments of λ DNA **(lane 1)**, MCF-7-2-2 (secondary nude mouse tumor, **lane 2**), 3T3 mcf.2.3 **(lane 3)**, cos M2-t1 **(lane 4)**, NIH 3T3 **(lane 5)**, NIH 3T3 + 2.5×10^{-3} μg of cos M2 DNA **(lane 6)**, 3T3 mcf.2.1 **(lane 7)**, 3T3 mcf.2.1-t1 **(lane 8)** and 3T3 mcf.2.1-t3 **(lane 9)** (see text and the legend to Table I for a description of the cell lines). The sizes of the *mcf*.2 *Eco*RI fragments are indicated.

Expression of mcf.2 in transformed NIH 3T3 cells and in human cell lines

Aliquots of poly(A)$^+$ RNA isolated from MCF-7, MCF-7-2, (nude mouse tumor), 3T3 mcf.2.1, control NIH 3T3 cells and three human cell lines, KG-1, K-562 and MIA PaCa-2 (see Materials and methods), were electrophoresed in a denaturing gel, blotted onto nitrocellulose and hybridized with a RNA probe derived from a cDNA clone (n121), as described in Materials and methods. A 2.7-kb transcript was detected in MCF-7-2 cells (Figure 3, lane 2), and a smaller 2.3-kb transcript was detected in 3T3 mcf.2.1 cells (lane 3). In MCF-7-2 cells, the 2.7-kb transcript was amplified 30- to 40-fold, as judged by densitometer scanning of the autoradiograms. Both transcripts were seen in the three human cell lines (lanes 6–8), although the 2.3-kb transcript was always more abundant than the 2.7-kb species. At least the 2.3-kb transcript was clearly seen in MCF-7 cells (lane 5); the 2.7-kb species was either absent or hardly detectable. The longer exposure required to detect the *mcf*.2 transcript in these cells also revealed some cross-hybridization with the 28S rRNA (4.7-kb band), also seen in the other cell lines, even NIH 3T3, under these conditions (data not shown). The probe did not hybridize with mouse transcripts in NIH 3T3 cells (lane 4).

Search for homology of mcf.2 with known oncogenes

A cDNA clone, pb51, hybridizing to the 4.5- and 3.3-kb *Eco*RI

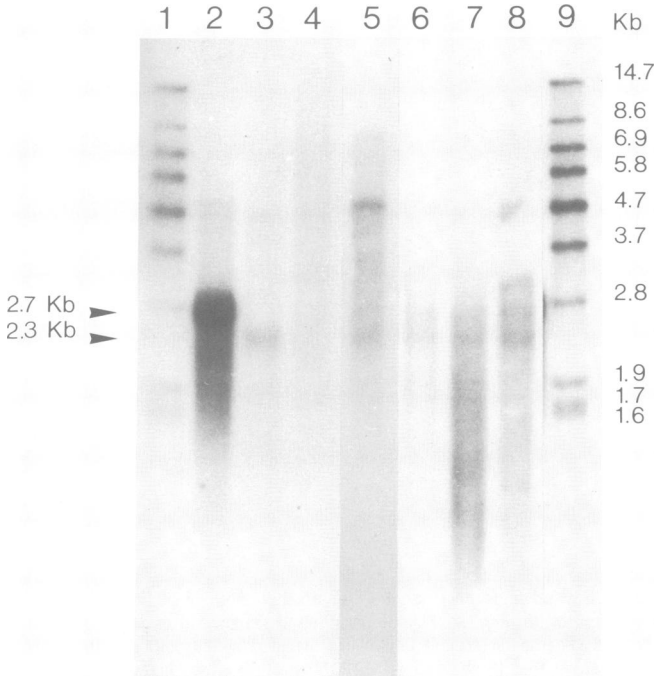

Fig. 3. Expression of *mcf*.2 in transformed and human cells. Poly(A)$^+$ RNA samples were prepared and electrophoresed under denaturing conditions as described under Materials and methods, transferred onto nitrocellulose and hybridized with the n121 probe. The various lanes contained: marker λ DNA fragments (*Ava*I + partial *Nar*I digest) **(lanes 1 and 9)**, 2 μg of MCF-7-2 RNA **(lane 2)**, 2 μg of 3T3 mcf.2.1 RNA **(lane 3)**, 10 μg of NIH 3T3 RNA **(lane 4)**, 5 μg of MCF-7 RNA **(lane 5)** and 5 μg each of KG-1 **(lane 6)**, K-562 **(lane 7)** and MIA PaCa-2 **(lane 8)** RNA, respectively. **Lane 5** (MCF-7) was a longer exposure of the blot, making the 4.7-kb band of rRNA more prominent (see Results). The sizes of the two *mcf*.2 transcripts are indicated on the left, and the sizes of the marker fragments are indicated on the right of the figure.

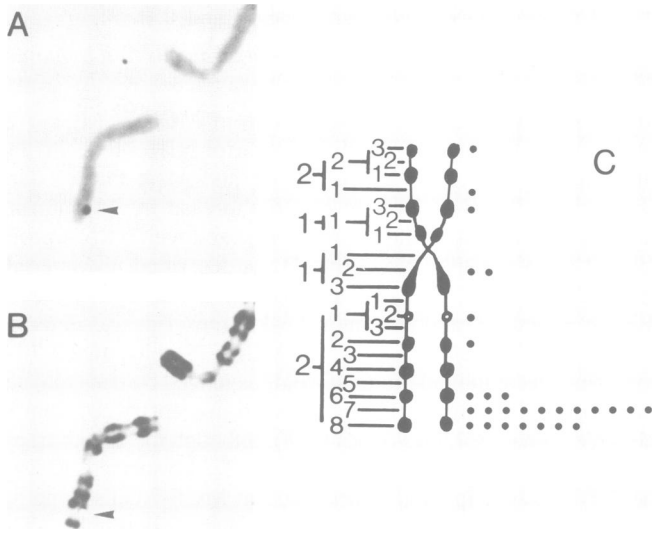

Fig. 4. *mcf*.2 maps at Xq27. The pb51 plasmid clone, containing a cDNA insert homologous to the 4.5- and 3.3-kb fragments of cos M2 shown in Figure 1B, was used for *in situ* hybridization on human metaphase chromosomes. **A** and **B**, typical hybridization pattern with Giemsa staining and R banding, respectively. **C**, detailed distribution of silver grains on 28 labeled X chromosomes.

Table II. Regional localization of *mcf*.2

Human−rodent somatic hybrid[a]	X segment contained in the hybrid[a]		Hybridization of X-specific probes[b]			
			St1	FIX	4.5-kb *mcf*.2	St14
Cer.S	pter	→ q11.2	−	−	−	−
58.6	pter	→ q21-23	−	−	−	−
GM 194	pter	→ q28	+	+	+	+
GO.4	p11	→ qter	+	+	+	+
PI.7.2	p22	→ qter	+	+	+	+
HRBC2	p22.3	→ qter	+	+	+	+
34.X	p22.3	→ qter	+	+	+	+
Cer.H	q11.2	→ qter	+	+	+	+
Anly.1	q12	→ qter	+	+	+	+
56.0	q21	→ qter	+	+	+	+
GM89	q22-24	→ qter	+	+	+	+
35.A1	q23	→ qter	+	−	−	+
63.R	q26	→ qter	+	+	+	+
GM 97	q26	→ qter	+	+	+	+

[a]The origin and the characteristics of the various human−rodent hybrid cell lines have been described (Oberlé *et al.*, 1986a).
[b]Results of Southern blot hybridizations of *Pst*I-digested human−rodent hybrid DNAs with the ^{32}P-labeled 4.5-kb *Eco*RI fragment of cos M2 DNA. The data for St1 (probe for the St1 locus), FIX (cDNA probe for the coagulation factor IX) and St14 (probe for the St14 locus) are in part from Oberlé *et al.* (1986a). (+) and (−), signal for X-specific hybridization, and no signal, respectively.

fragments of cos M2, was hybridized under suitable conditions of stringency to a series of oncogenes as described previously (Fasano *et al.*, 1984) and to a cDNA clone carrying *mcf*.3 coding sequences (Birchmeier *et al.*, 1986). None of the oncogenes thus tested showed hybridization with the *mcf*.2 sequence.

Chromosomal localization of mcf.2

The chromosomal localization of the *mcf*.2 transforming locus was determined by *in situ* hybridization on normal human chromosomes, using as a probe the cDNA clone pb51. The specific location of silver grains was assigned by chromosome banding after *in situ* hybridization and autoradiography, and is shown in Figure 4A and B. Analysis of 130 metaphase spreads showed that 14.7% of the silver grains were localized on the X chromosomes. Figure 4C represents a detailed distribution of silver grains on 28 labeled X chromosomes. About 75% of the grains (21/28) were clustered on the Xq26, Xq27 and Xq28 bands; the maximum number of grains was observed on the Xq27 band, which was therefore the most probable localization for *mcf*.2. To further document this result, the 4.5-kb *Eco*RI genomic fragment of cos M2 was used in Southern blots against several human DNAs (Figure 5A): a normal male (46,XY) (lane 3), a normal female (46,XX) (lane 4), and a male with three supernumerary X chromosomes (49,XXXXY) (lane 5). The intensity of the *Eco*RI fragment hybridizing with the 4.5-kb probe varied as a function of the number of copies of the X chromosome present in the DNA sample. The same blot was hybridized with the left hand side 2.4-kb *Eco*RI fragment, purified from cos H4 DNA. Examination of this autoradiogram (Figure 5B) showed no variation in the intensities of the 2.4-kb bands for the different DNA preparations. This result strongly suggested that this 2.4-kb *Eco*RI fragment, and probably also part of its flanking sequences, were not located on the X chromosome. *In situ* hybridization on normal human chromosomes localized in fact

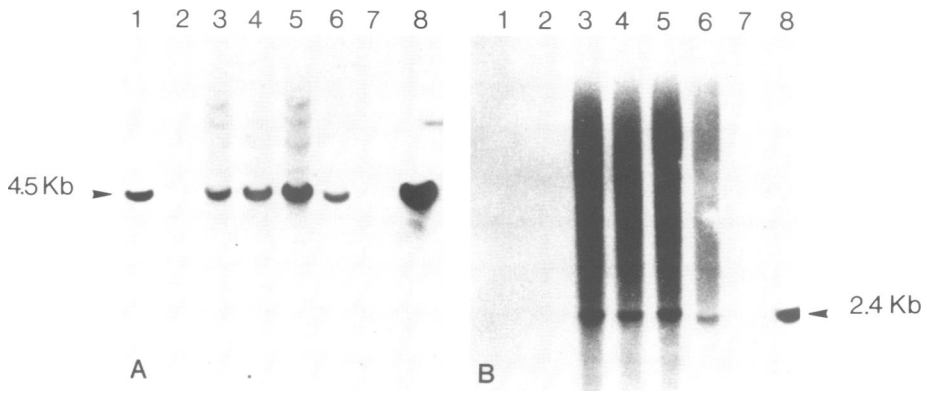

Fig. 5. Hybridization of *mcf*.2 to human DNA samples showing the proportionality to the number of X chromosomes. **(A)** A blot containing *Eco*RI-digested DNAs (20 μg) from a normal male (46,XY) **(lane 3)**, a normal female (46,XX) **(lane 4)** and a male with three supernumerary X chromosomes (49,XXXXY) **(lane 5)** was hybridized with the 4.5-kb *Eco*RI fragment of cos M2. Several lanes of control *Eco*RI-digested DNAs were also included: NIH 3T3 (10 μg) + 1×10^{-3} μg of cos M2 **(lane 1)**, NIH 3T3 (10 μg) **(lanes 2** and **7)**, MCF-7 (5 μg) **(lane 6)** and MCF-7-2-2 (5 μg) **(lane 8)**, respectively. **(B)** The same blot was subsequently dehybridized and rehybridized with the 2.4-kb fragment of cos H4 (see Figure 1 for details on the fragments). The relative order of the samples was the same as in **A**. Although this 2.4-kb fragment did not appear to contain repetitive sequences when hybridized with total human DNA, the background hybridization seen here suggests that it may in fact contain low repetition sequences.

this 2.4-kb fragment on the long arm of chromosome 3, at q26 (data not shown). The *mcf*.2 locus therefore appears to have been created by a rearrangement between two different non-synthenic portions of the human genome.

In addition, the regional localization of *mcf*.2 at Xq27 was confirmed by Southern blot hybridization of the 4.5-kb *Eco*RI fragment of cos M2 to a panel of 14 human−rodent hybrid cell DNAs, as shown in Table II. This regional localization was in close agreement with the data obtained by *in situ* hybridization (see above). Interestingly, a comparative analysis of the mapping of *mcf*.2 and of other X-specific probes, tested previously on the same panel of hybrids (Oberlé *et al.*, 1986a, and Table II), indicated that *mcf*.2 was very close to the human anti-hemophilic factor IX. Indeed, *mcf*.2 was missing from the 35.A1 hybrid, which lacks a very small portion of the X chromosome around the factor IX locus.

Discussion

We report here the molecular cloning and chromosomal localization of the *mcf*.2 transforming sequence. This gene was initially isolated from a nude mouse tumor obtained after injection of NIH 3T3 cells co-transfected with DNA from MCF-7, a human mammary carcinoma cell line, and a plasmid carrying the G418 antibiotic resistance gene. Partial cloning of *mcf*.2 was reported previously (Fasano *et al.*, 1984). We isolated another portion of the *mcf*.2 locus from the DNA of the same nude mouse tumor, using cosmid cloning, and found that a transforming activity was present in one of these clones, outside the previously published region. One individual cosmid clone, cos M2, was able to transform NIH 3T3 cells in a classical focus assay as well as in the tumorigenicity assay used to detect the *mcf*.2 transforming gene. Preliminary mapping of the transcribed portions of *mcf*.2 has shown that the cDNA clones isolated to date were contained within three *Eco*RI fragments of cos M2.

We localized a segment of *mcf*.2 carrying the transforming activity to the X chromosome at q27, near the X fragile site (Sutherland and Ashford, 1979). *mcf*.2 appears to be the only known transforming gene mapping at this location, though proto-oncogenes have been mapped at other locations on the X chromosome: the c-Ha-*ras*.2 pseudogene (Miyoshi *et al.*, 1984), on the long arm at the centromeric-proximal (to q26) region (O'Brien *et al.*, 1983) and a member of the *raf* family (hA-*raf*-1), in the Xp21→Xq11 region (Huebner *et al.*, 1986). In addition, using relatively low stringency hybridization of Southern blots, we did not detect any homology between a *mcf*.2 cDNA clone, located within the transforming region of cos M2, and all the other oncogenes tested. We therefore believe that *mcf*.2 may represent a new oncogene.

Using this co-transfection/tumorigenicity assay, another gene has been isolated previously (Fasano *et al.*, 1984): this oncogene was created through a rearrangement of a normal cellular gene, which in all likelihood occurred during gene transfer (Birchmeier *et al.*, 1986). Rearrangements following transfection events were also observed for the *mas* (Young *et al.*, 1986) and *ret* (Takahashi *et al.*, 1985) oncogenes. A similar recombination event appears to have created the *mcf*.2 locus. Two lines of results support this hypothesis. Partial cloning of the *mcf*.2 allele from MCF-7 DNA, and restriction enzyme analysis of these cosmid clones, have shown that the *mcf*.2 map of MCF-7 was not co-linear with that of the tumoral gene, starting ~6 kb upstream from the transforming portion. Second, the results of the hybridizations of the 2.4-kb fragment, located in the left portion of the *mcf*.2 locus, against a set of human DNAs containing 1−4 X chromosomes, as well as the results of the *in situ* hybridization of this clone to human chromosomes, strongly supported the assumption that the *mcf*.2 locus, as present in the nude mouse tumor, was created by a recombination event. In any case, the region shown as containing coding sequences, and a transforming capacity, was not rearranged, as overlapping phage clones with the same organization were cloned from a non-tumoral DNA library. This makes it difficult to investigate the role of the non-X sequences present in the *mcf*.2 locus. Complete cDNA cloning and sequencing will allow the determination of whether these sequences participate in the transforming activity.

Northern blot hybridization detected two transcripts, of 2.7 and 2.3 kb, in human tumor cells; in contrast, the two types of transformed cells contained a single transcript. As mentioned above, availability of complete cDNA clones will allow mapping of the transcripts along the gene, and elucidation of the mechanism of their generation.

Our results provide evidence that *mcf*.2 may be a new oncogene, and that its transforming region maps to the X chromosome, near the fragile site. The determination of the complete nucleotide sequence, and of the mode of activation of *mcf*.2,

should allow us to understand its putative role in human malignancies. The isolation of cosmid clones corresponding to the normal and MCF-7 alleles of *mcf*.2 will help us to elucidate the possible contributions of amplification and rearrangement to the activation of this gene.

Finally, the location of *mcf*.2 on the X chromosome may prove useful for cytogeneticists in studies concerning the X fragile site. Indeed, *mcf*.2 and the anti-hemophilic factor IX seem physically very close, and this last locus is already used, in conjunction with cytogenetic analysis, for genetic counselling of the fragile X syndrome (Oberlé *et al.*, 1986b).

Materials and methods

Transformation of NIH 3T3 cells and tumorigenicity assays

NIH 3T3 cells and the pT24 Ha-*ras*.1 plasmid were gifts of M.Wigler (Cold Spring Harbor Laboratory, NY). Focus assays were performed as described previously (Perucho *et al.*, 1981). Tumorigenicity assays were performed following the procedure established in M.Wigler's laboratory and described previously (Fasano *et al.*, 1984). The pKOneo plasmid was a gift of D.Hanahan (Cold Spring Harbor Laboratory, NY). Briefly, 2.5 μg of cloned DNA and/or 30 μg of cellular DNA plus 0.3 μg of pKOneo plasmid were co-transfected into 7×10^5 NIH 3T3 cells. Selection by the G418 antibiotic (0.5 g/l) was applied 20 h after transfection, and resistant cells were grown into colonies. Confluent cell colonies were harvested, pooled and $\sim 1 \times 10^7$ cells were injected s.c. into 4- to 5- weeks-old athymic Swiss mice (Iffa Credo, Lyon, France). Tumors developing at the site of inoculation were removed from sacrificed animals. Cell lines were derived from tumor fragments. All the cells were grown in Dulbecco's-modified Eagle medium, supplemented with 10% newborn calf serum and antibiotics, when appropriate.

Molecular cloning

Cosmid libraries were constructed from *Eco*RI partial digests of DNA extracted from MCF-7 cells and from the MCF-7-2-2 nude mouse tumor. DNA fragments 35–45 kb long were purified by velocity centrifugation in a 15–40% sucrose gradient, and ligated to *Eco*RI-cleaved, phosphatase-treated pHC79 DNA vector (Hohn and Collins, 1980). Ligated material was packaged into phage particles using an *in vitro* packaging kit (Amersham) and introduced into 490A *Escherichia coli* cells. The λ phage library, constructed from peripheral blood lymphocyte DNA, was a gift of B.Jordan (Centre d'Immunologie, Marseille). A cDNA library was constructed as described previously (Huynh *et al.*, 1984).

Samples of non-amplified libraries were screened under stringent conditions (6 × SSC, 1 × Denhardt's solution, 20 μg/ml of denatured salmon sperm DNA, at 72°C for 20 h) by hybridization with appropriate ^{32}P-labeled purified DNA fragments, followed by three washes in 2 × SSC/0.1% SDS.

Southern blot hybridization

Southern blot hybridizations of nitrocellulose filters, under stringent or non-stringent conditions, of electrophoresed cloned or genomic DNAs were performed as described previously (Fasano *et al.*, 1984). For determination of the regional localization of *mcf*.2, *Pst*I-digested DNAs from human–rodent hybrid cell lines were blotted onto diazobenzyloxymethyl paper and hybridizations were performed as described previously (Oberlé *et al.*, 1986a). Labeling of the DNA probes was performed either by nick-translation or by oligonucleotide labeling (Feinberg and Vogelstein, 1983).

RNA preparation and Northern blot hybridization

Total cellular RNA was prepared from NIH 3T3, 3T3 mcf.2.1, MCF-7, MCF-7-2 cells and three human cell lines, KG-1 [acute myelogenous leukemia, (Koefler and Golde, 1978)], K-562 [chronic myelogenous leukemia, (Lozzio and Lozzio, 1975] and MIA PaCa-2 [pancreatic carcinoma, (Yunis *et al.*, 1977)], by lysis in guanidinium isothiocyanate, followed by fractionation over a cesium chloride cushion as described (Maniatis *et al.*, 1982). Poly(A)$^+$ RNAs were purified by oligo(dT)–cellulose (Collaborative Research) chromatography as described (Aviv and Leder, 1972); absence of degradation was checked by electrophoresis through vertical slab gels containing methylmercuric hydroxide (Alfa Chemicals) as described (Bailey and Davidson, 1976). Denatured poly(A)$^+$ RNA samples, together with marker DNA fragments, were electrophoresed through 1% agarose gels containing formaldehyde (Thomas, 1980), transferred to nitrocellulose and hybridized with a ^{32}P-labeled RNA probe, obtained by transcription of a 350-bp *mcf*.2 cDNA clone (n121) inserted into a SP6 vector (Melton *et al.*, 1984). Pre-hybridizations and hybridizations were performed in 5 × SSPE buffer (0.9 M NaCl, 0.05 M sodium phosphate pH 7.7 and 0.4 mM EDTA) containing 50% formamide, 200 μg/ml sonicated salmon sperm DNA, 5 × Denhardt's solution and 0.1% SDS at 42°C for 20 h. Filters were then washed at 42°C twice in 2 × SSC/0.1% SDS and twice in 0.4 × SSC/0.1% SDS.

In situ chromosome hybridization

Plasmids pb51, containing a cDNA insert, and pmcf.2-4, containing the 2.4-kb *Eco*RI genomic fragment (see Figure 1B), both inserted in pUC9, were tritium-labeled by nick-translation to specific activities of $1-1.3 \times 10^8$ d.p.m./μg. Phytohemagglutinin-stimulated lymphocytes from a normal female were cultured for 72 h. 5-Bromodeoxyuridine was added during the last 7 h of culture (final concentration: 30–60 μg/ml) to ensure a good post-hybridization chromosomal banding. Metaphase cells were hybridized using a probe concentration of 2 μg/ml of hybridization mixture. Slides were covered with Kodak NTB 2 nuclear track emulsion, and exposed for 12 days at 4°C. After development, the chromosome spreads were first stained with buffered Giemsa solution and metaphases were photographed. R-banding was then performed by the fluorochrome–photolysis–Giemsa method, and metaphases re-photographed before analysis. The technique has been described previously (Mattéi *et al.*, 1985).

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