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Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to β-thromboglobulin

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Melanoma growth stimulatory activity (MGSA) is a mitogenic polypeptide secreted by Hs294T human melanoma cells. Comparison of the N-terminal sequences of the 13 and 16 kd MGSA species with the cDNA sequence revealed that the mature form of human MGSA is maximally 73 amino acids long. Expression of the cDNA in mammalian cells results in the secretion of this peptide with mitogenic activity. MGSA is structurally related to the platelet-derived β-thromboglobulin and to several other polypeptides. These factors may constitute a family of growth factors. MGSA mRNA was detected in a variety of cell types. The level of MGSA mRNA in melanoma cells is strongly elevated by treatment with MGSA. MGSA is the gene product of a recently detected gene *gro*. The gene was mapped to chromosome 4 (region q13→q21). This same region also contains genes for two of the structurally related factors, for c-*kit*, a receptor for an as yet unidentified ligand, and for 'piebald trait', an inherited skin pigmentation disorder.

Key words: Melanoma growth stimulatory activity/ β-thromboglobulin/growth factor

Introduction

A variety of growth factors can affect the proliferation and the phenotypic appearance of cells. The endogenous production of some growth factors can strongly influence the establishment of the transformed character. The endogenous growth factors that are best studied for these effects are the platelet-derived growth factors, bombesin, transforming growth factor (TGF)-α and -β, basic fibroblast growth factor and the insulin-like growth factors I and II (for review see Goustin *et al.*, 1986). The role of these agents in the autonomous growth of the tumor cell may be complex and may vary among and within the types of transformed cells. It is now generally accepted that the interaction of these growth factors with the cells can take place in an autocrine fashion (Sporn and Todaro, 1980; Sporn and Roberts, 1985).

A less well known growth factor is the melanoma growth stimulatory activity (MGSA). This activity was initially found in the culture medium of the Hs294T human malignant melanoma cell line (Richmond *et al.*, 1982a,b, 1983, 1985). Biochemical and immunocytochemical studies have revealed that MGSA is secreted by ~70% of the primary cell cultures from human melanoma biopsies and by a majority of benign nevus cells with chromosomal abnormalities. In contrast, newly established serum-free cultures of benign nevus cells with a normal karyotype are negative for MGSA production. These data may therefore suggest that deregulation of MGSA production is associated with events involved in tumor progression (Richmond *et al.*, 1986; Richmond and Thomas, 1988).

It is possible that MGSA functions as an autocrine growth factor for malignant melanoma cells. When nanogram quantities of purified MGSA are added to low density, serum-free cultures of the Hs294T malignant melanoma cells, a significant increase in the growth rate becomes apparent (Richmond *et al.*, 1982a,b; Richmond and Thomas, 1986). Monoclonal antibodies to MGSA markedly inhibit the growth of Hs294T cells grown in serum-free medium and the growth response of cells to exogenous MGSA (Lawson *et al.*, 1986). The involvement of MGSA in cell proliferation may not be restricted to malignant melanoma cells since immunoreactive MGSA is present in the epidermis of the skin and in a number of tissues with proliferative disorders (Richmond and Thomas, 1988). Also, immortalized rodent fibroblasts are mitogenically stimulated by MGSA (Richmond *et al.*, 1983).

Biochemical studies have shown that MGSA bioactivity is associated with acid- and heat-stable polypeptides ranging from 9 to 26 kd based on polyacrylamide gel electrophoresis. However, the majority of activity is found in MGSA proteins with an apparent mol. wt of 13 and 16 kd (Richmond and Thomas, 1986). Reduction with dithiothreitol destroys MGSA bioactivity but does not significantly alter the mobility in polyacrylamide gels, suggesting that intrachain disulfide bridge formation is required for the biological activity (Richmond and Thomas, 1986). Recently a high yield purification method for MGSA was developed by sequential use of Biogel P-30 gel filtration, heparin-sepharose and reverse phase high pressure liquid chromatography (Thomas and Richmond, 1988).

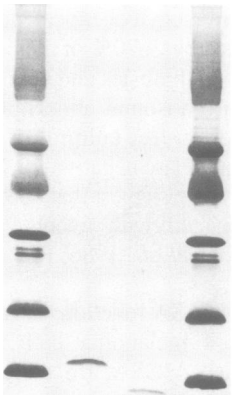
The purification of sufficient quantities of MGSA from the Hs294T melanoma cell culture medium allowed the determination of the amino acid sequence of the NH_2-terminal 34 residues. Expresion of this cDNA in mammalian cells confirmed the mitogenic nature of the secreted factor. Northern hybridizations using the MGSA cDNA revealed the expression of MGSA mRNA in various tumor cells. Treatment of melanoma cells with MGSA induced high levels of MGSA mRNA. The human MGSA gene was mapped to chromosome 4 (region q13→q21), a

region that also contains the genes for the c-*kit* receptor, for platelet factor 4 and for the interferon-γ induced factor γIP-10.

Results

The amino-terminal polypeptide sequence

MGSA was obtained from conditioned serum-free medium of the melanoma cell line Hs294T. Two MGSA species with an apparent mol. wt of 13 and 16 kd were purified by gel filtration, heparin-sepharose and reverse phase high performance liquid chromatography (Thomas and Richmond, 1988). These two peptides, which retained mitogenic activity (Thomas and Richmond, 1988), were then subjected to direct N-terminal amino acid sequencing (Fisher and Spiess, 1987). This analysis revealed a 34 residue long N-terminal sequence for the 13 kd MGSA. Similar analysis of the fraction containing the 16 kd species revealed an N-terminus, that was in agreement with the 13 kd species (Figure 1). However, the amount of observed peptide was much lower than expected on the basis of the amount subjected to sequence analysis. Thus, the peptide identified in the fraction containing the 16 kd species may represent a minor component of this fraction.



16,000 Mr form of MGSA

NH$_2$-X-X-val-ala-thr-glu-leu-arg-X-gln-X-leu

13,000 Mr form of MGSA

NH$_2$-ala-ser-val-ala-thr-glu-leu-arg-cys-gln-cys-leu-gln-thr-leu-gln-gly-ile-his-pro-lys-asn-ile-gln-ser-val-asn-val-lys-ser-pro-gly-pro-his

Fig. 1. Amino terminal sequences directly determined on the purified 13 and 16 kd MGSA species. The electrophoretic separation of the two reduced MGSA polypeptides (16 kd, **lane 2**; 13 kd, **lane 3**) on a SDS–denaturing gel (linear gradient of 12–18% acrylamide) is shown. The samples were reduced before loading.

Isolation of cDNAs for MGSA

Two long synthetic oligonucleotides were designed on the basis of the N-terminal sequence for the 13 kd MGSA species, using the codon bias observed in human mRNAs (Grantham *et al.*, 1981). Initially, two cDNA libraries, one derived from human placenta mRNA and the other from the human melanoma cell line 3728, were screened using the long oligonucleotides as hybridization probes under low stringency hybridization conditions. Several cDNAs hybridized with either probe. These cDNAs were then also hybridized to a third oligonucleotide, that corresponds to amino acids 14–34 of mature MGSA. This analysis led to the isolation of one MGSA cDNA from the placenta cDNA library. This cDNA was incomplete since the sequence coding for the N-terminal six amino acids was lacking. To isolate additional and complete cDNAs, a cDNA library was prepared using mRNA from the Hs294T melanoma cell line that was originally used for isolation of the MGSA polypeptides. Screening of this library with the previously isolated partial MGSA cDNA insert revealed the presence of several cDNAs, all of which had a length of ~1050 bp.

The sequence of the MGSA cDNA and precursor polypeptide

The known amino acid sequence of the N-terminus of MGSA established the reading frame in the cDNA sequence (Figure 2). The coding sequence ends at nt 338 with a TGA stop

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                                                  10                                  20
                        MetAlaArgAlaAlaLeuSerAlaAlaProSerAsnProArgLeuLeuArgValAlaLeuLeuLeuLeuLeuLeuValAlaAla
   1 CCCGCCTGCTGAGCCCCATGGCCCGCGCTGCTCTCTCCGCCGCCCCCAGCAATCCCCGGCTCCTGCGAGTGGCACTGCTGCTCCTGCTCCTGGTAGCCGC

       30                                  40                                  50                                  60
     GlyArgArgAlaAlaGlyAlaSerValAlaThrGluLeuArgCysGlnCysLeuGlnThrLeuGlnGlyIleHisProLysAsnIleGlnSerValAsn
 101 TGGCCGGCGCGCAGCAGGAGCGTCCGTGGCCACTGAACTGCGCTGCCAGTGCTTGCAGACCCTGCAGGGAATTCACCCCAAGAACATCCAAAGTGTGAAC

                                  70                                  80                                  90
     ValLysSerProGlyProHisCysAlaGlnThrGluValIleAlaThrLeuLysAsnGlyArgLysAlaCysLeuAsnProAlaSerProIleValLysLys
 201 GTGAAGTCCCCCGGACCCCACTGCGCCCAAACCGAAGTCATAGCCACACTCAAGAATGGGCGGAAAGCTTGCCTCAATCCTGCATCCCCCATAGTTAAGA

                 100
       IleIleGluLysMetLeuAsnSerAspLysSerAsn
 301 AAATCATCGAAAAGATGCTGAACAGTGACAAATCCAACTGACCAGAAGGGAGGAGGAAGCTCACTGGTGGCTGTTCCTGAAGGAGGCCCTGCCCTTATAG

 401 GAACAGAAGAGGAAAGAGAGACACAGCTGCAGAGGCCACCTGGATTGTGCCTAATGTGTTTGAGCATCGCTTAGGAGAAGTCTTCTATTTATTTATTTAT

 501 TCATTAGTTTTGAAGATTCTATGTTAATATTTTAGGTGTAAAATAATTAAGGGTATGATTAACTCTACCTGCACACTGTCCTATTATATTCATTCTTTTT

 601 GAAATGTCAACCCCAAGTTAGTTCAATCTGGATTCATATTTAATTTGAAGGTAGAATGTTTTCAAATGTTCTCCAGTCATTATGTTAATATTTCTGAGGA

 701 GCCTGCAACATGCCAGCCACTGTGATAGAGGCTGGCGGATCCAAGCAAATGGCCAATGAGATCATTGTGAAGGCAGGGGAATGTATGTGCACATCTGTTT

 801 TGTAACTGTTTAGATGAATGTCAGTTGTTATTTATTGAAATGATTTCACAGTGTGTGGTCAACATTTCTCATGTTGAAACTTTAAGAACTAAAATGTTCT

 901 AAATATCCCTTGGACATTTTATGTCTTTCTTGTAAGGCATACTGCCTTGTTTAATGGTAGTTTTACAGTGTTTCTGGCTTAGAACAAAGGGGCTTAATTA

1001 TTGATGTTTTCATAGAGAATATAAAAATAAAGCACTTATAGAAAAAAAAAAAAAAAAAAAA
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Fig. 2. MGSA cDNA sequence and derived amino acid sequence. The N-terminus of mature MGSA as determined from direct peptide sequencing is shown by the arrow.

codon, and is followed by the 3′ untranslated region including part of the poly(A) tail. This 3′ untranslated region contains several stretches of AT-rich sequences. Such sequences with a consensus ATTTA have been found in the cDNAs of certain lymphokines, cytokines and proto-oncogenes, and are presumably involved with selective degradation of transiently expressed mRNAs (Shaw and Kamen, 1986).

The presumed initiator codon is at position 17 and the deduced polypeptide sequence is therefore 107 amino acids long. The initiator methionine is followed by a very hydrophobic sequence extending to amino acid 28. Comparison with other known signal sequences (Von Heijne, 1986) suggests that this N-terminal amino acid sequence corresponds to a signal peptide. Proteolytic cleavage occurs presumably following the Gly at position 34 since the amino acid sequence starting at position 35 corresponds to the directly determined N-terminal sequence of the 13 kd MGSA. Four cysteine residues are present in the mature MGSA sequence. These may be involved in the formation of two intrachain disulfide bridges. No potential N-glycosylation site (Asn−X−Ser or −Thr) are present.

Structural similarity with other polypeptides

The deduced amino acid sequence for the MGSA precursor shows extensive similarity with human platelet basic protein (PBP; Holt *et al.*, 1986), a precursor form of β-thromboglobulin (β-TG; Begg *et al.*, 1978) and connective tissue activating peptide III (CTAP-III), also known as low affinity platelet factor-4 (Castor *et al.*, 1983). CTAP-III is a mitogenic polypeptide that is stored in platelets and released upon thrombin-induced platelet aggregation (Castor *et al.*, 1983). Additional sequence similarity was also found with the heparin binding platelet factor-4 (PF-4) (Deuel *et al.*, 1977; Poncz *et al.*, 1987), an inhibitor of collagenase (Hiti-Harper *et al.*, 1978), and with an interferon-γ induced peptide (γIP10; Luster *et al.*, 1985). Also, peripheral blood leukocytes induced for mitosis by Staphylococcus enterotoxin A synthesize an mRNA called 3-10C which encodes a protein with amino acid sequence similarity to MGSA (Schmid and Weissman, 1987). In addition, Sugano *et al.* (1987) and Bedard *et al.* (1987) recently reported the cDNA sequence designated 9E3 or pCEF-4 for yet another polypeptide with sequence similarity to these members of the β-thromboglobulin family. The corresponding mRNA levels for this 9E3 (or pCEF-4) derived protein in chicken embryo fibroblasts are transiently induced following serum stimulation of quiescent cells, and there is a strongly elevated expression upon transformation with Rous sarcoma virus. In all these related proteins the positioning of the four cysteine residues

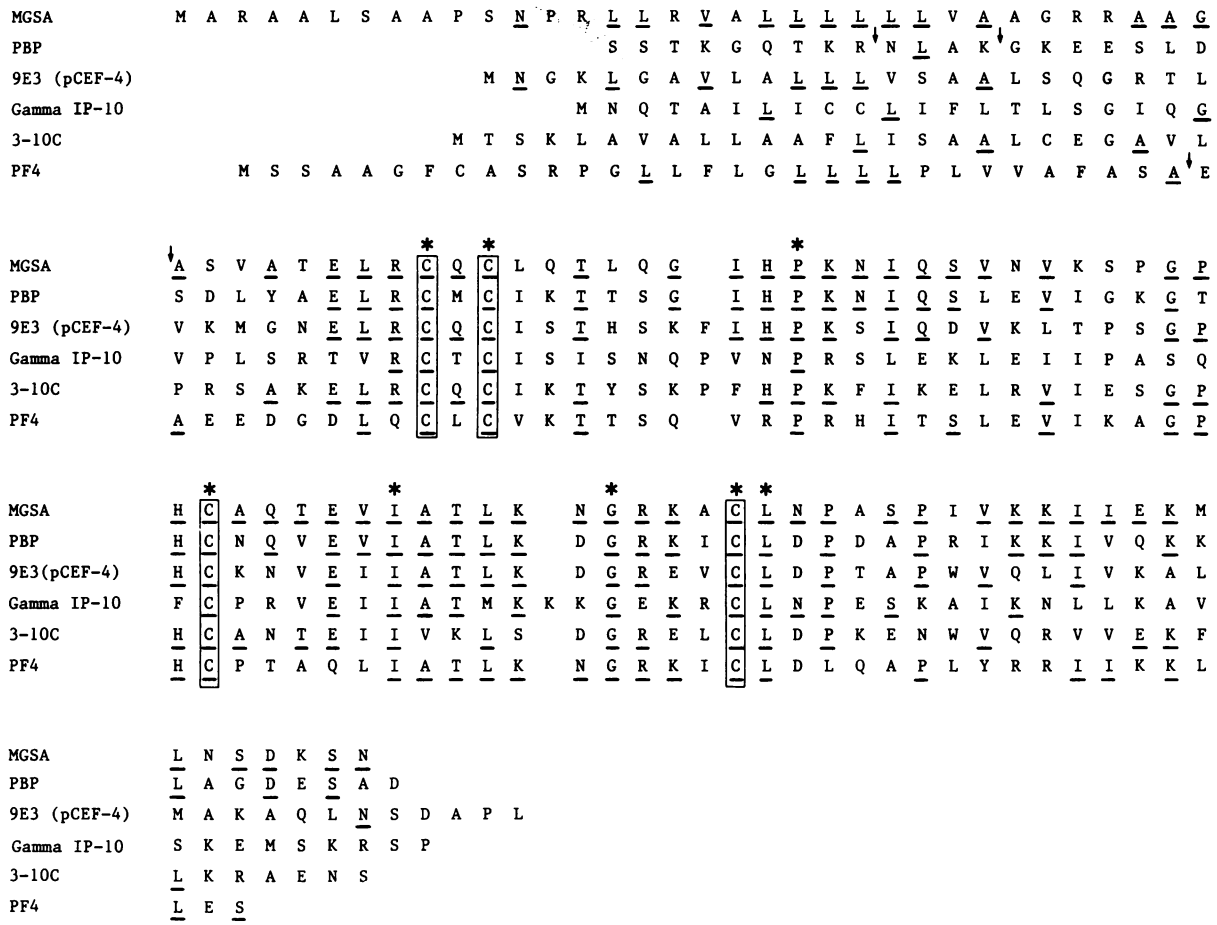

Fig. 3. Structural similarities between several polypeptide sequences. The MGSA precursor sequence is compared with the sequence for PBP (Holt *et al.*, 1986) and for PF4 (Deuel *et al.*, 1977; Poncz *et al.*, 1987). The amino acid sequences for 9E3 (pCEF4) (Sugano *et al.*, 1987; Bedard *et al.*, 1987), γIP-10 (Luster *et al.*, 1985), 3-10C (Schmid and Weissman, 1987) and PF4 (Poncz *et al.*, 1987) were derived from cDNAs. The underlined residues show identity with the MGSA sequence. The asterisks designate identity for all six sequences. The Cys-residues are boxed. The arrows indicate proteolytic cleavage sites.

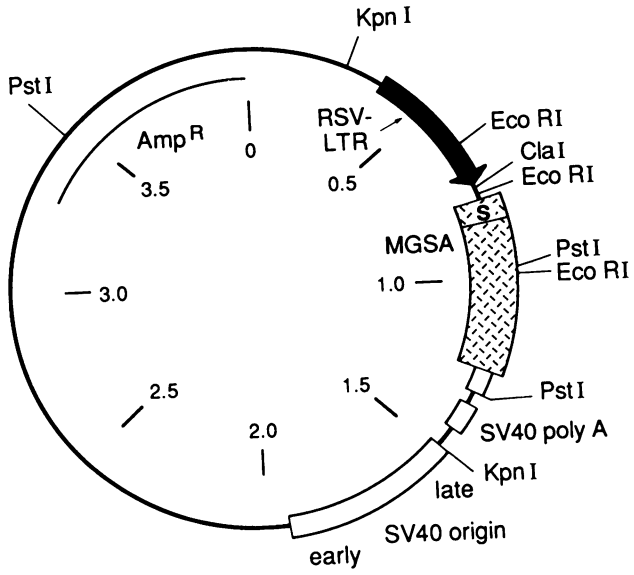

Fig. 4. Schematic diagram of the pMGSA 5-2/3 MGSA expression vector. The Rous sarcoma virus long terminal repeat with the direction of transcription is indicated with a black arrow. The S within the MGSA sequence (boxed) represents the signal peptide sequence. SV40 poly(A) marks the DNA segment with the polyadenylation signal. The distances in kilobases are marked in the center.

Table I. [^{3}H]Thymidine incorporation into Hs294 T cells following treatment with medium from transfected cells. The data are expressed as percent of the control level using medium from mock-transfected cells (± SD)

Mock infected cell population		100%	(20%)
1% fetal bovine serum		217%	(19%)
Non-selected transfected cell population 2.4 × diluted		236%	(19%)
Stable clone 11	10 × diluted	203%	(22%)
	100 × diluted	123%	(7%)
Stable clone 27	10 × diluted	260%	(13%)
Stable clone 50	10 × diluted	208%	(19%)
	100 × diluted	149%	(36%)
Stable clone 57	10 × diluted	237%	(32%)
	100 × diluted	258%	(6%)

is conserved (Figure 3). The positions of the two intrachain disulfide bridges have been characterized in the case of β-thromboglobulin (Begg *et al.*, 1978). By homology, it is likely that the MGSA protein contains disulfide bridges between the first and third, and between the second and fourth cysteines. While our work was ongoing, Anisowicz *et al.* (1987) reported the isolation of a cDNA named *gro*, which is transiently expressed following serum stimulation. The deduced amino acid sequence of the human *gro* polypeptide is identical to the MGSA precursor polypeptide.

Expression of the cDNA

In order to verify that the obtained cDNA encoded a factor with mitogenic activity, we expressed this cDNA in mammalian cells. The expression plasmid contained the coding sequence of the MGSA cDNA under the transcriptional control of the Rous sarcoma virus long terminal repeat. This

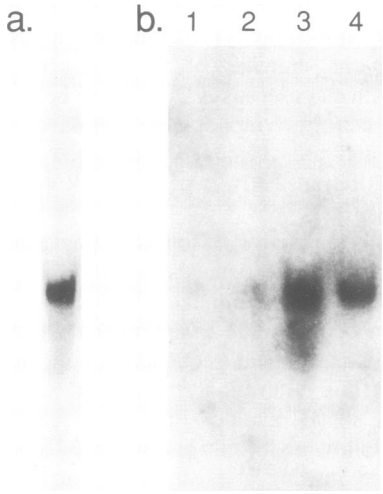

Fig. 5. MGSA mRNA detected by Northern hybridization of Hs294T melanoma cell mRNA (**a**) MGSA mRNA in subconfluent cell cultures. (**b**) Comparison of mRNA levels in cells not treated (**lanes 1** and **2**) or treated with MGSA (**lanes 3** and **4**). The cells in lanes 2 and 4 received a 4 h incubation with cycloheximide (see Materials and methods).

plasmid, shown in Figure 4, was transfected into human kidney cells (Eaton *et al.*, 1986) and the medium of the transfected cells was assayed for mitogenic activity on the Hs294T melanoma cells by measuring the incorporation of [^{3}H]thymidine (Richmond and Thomas, 1986). These cells are responsive to MGSA, yet do not respond to most currently characterized growth factors (Richmond *et al.*, 1982, 1983; Richmond and Thomas, 1988). In this assay, the increase in DNA synthesis correlates well with the mitogenic response, determined as cell number (Richmond and Thomas, 1988). The relatively high level of [^{3}H]-thymidine incorporation in the cells in the absence of exogenous MGSA or bovine serum is presumably due to the endogenous production of MGSA and possibly other factors. It has also been shown that the maximal increase in DNA synthesis, observed in the presence of 0.06 ng/ml or 6 ng/ml purified MGSA, is similar to the effect of 1% fetal bovine serum on these cells (Richmond and Thomas, 1988). The conditioned medium of the cells, transfected with the MGSA expression plasmid induced a level of DNA synthesis that was significantly elevated above the control level seen with the mock transfected cells and was comparable to the increase scored with fetal bovine serum (Table I). These data are thus in agreement with the fact that MGSA is encoded by the isolated cDNA.

mRNA for MGSA

Northern hybridizations revealed that there is one species of mRNA for MGSA in the Hs294T cells. This mRNA had a size of 1.1−1.2 kb and could be detected both in total RNA and in the polyadenylated mRNA fraction (Figure 5).

It has recently been shown that the expression of TGF-α in fresh keratinocytes is strongly increased following treatment of the cells with TGF-α itself (Coffey *et al.*, 1987). Such auto-inducible stimulation was also found in the case of platelet-derived growth factor (PDGF), since treatment of fibroblasts with PDGF increases the level of PDGF A-chain mRNA (Paulsson *et al.*, 1987). We tested whether

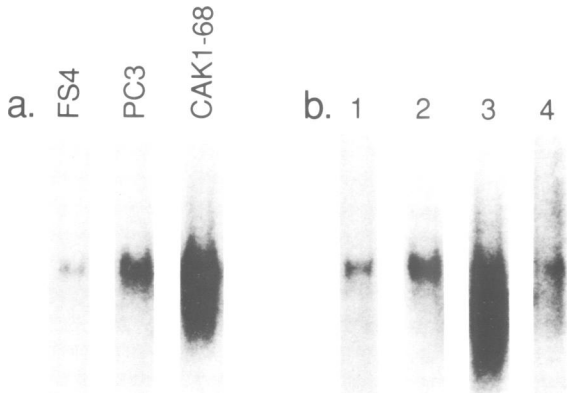

Fig. 6. Detection of MGSA mRNA in several cell sources (**a**) shows FS4 foreskin fibroblasts, PC3 prostate carcinoma cells and CAK1-68 renal carcinoma cells. (**b**) Shows examples of squamous carcinoma biopsies (**lanes 1** and **2**) and adenocarcinoma biopsies (**lanes 3** and **4**). For each sample, 3 μg of poly $(A)^+$ RNA were loaded on the gel. After transfer to nitrocellulose and hybridization the Northerns were exposed to Kodak XAR film for 48 h.

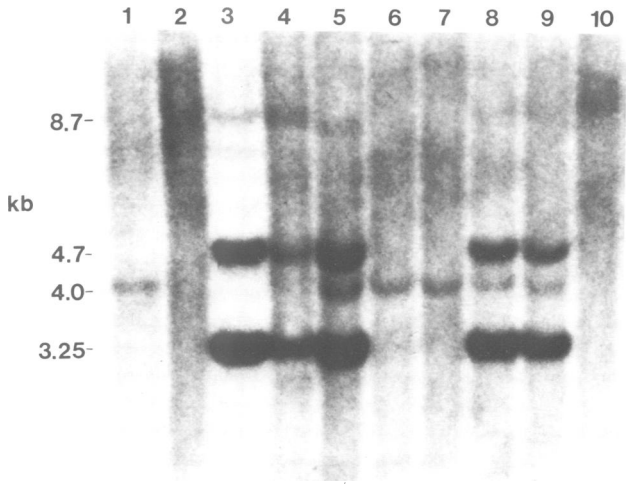

Fig. 7. Southern blot of *Eco*RI digested DNA from: **lane 1**, Chinese hamster cell line V79/380-6; **lane 2**, mouse liver; **lane 3**, human lymphoblastoid cell line; **lanes 4** and **10**, mouse × human hybrids; **lanes 5–9**, Chinese hamster × human hybrids. The sizes of the three human fragments and of the cross-hybridizing Chinese hamster fragment are indicated. The hybrid lines in lanes 4, 5, 8 and 9 were positive for all three human fragments and the other hybrids were negative.

MGSA can similarly induce its own synthesis. Northern hybridizations indicated that treatment of the Hs294T melanoma cells with MGSA at 3 ng/ml resulted in an increase of the MGSA mRNA level of at least 10-fold (Figure 5). The MGSA mRNA levels following MGSA treatment could not further be increased by cycloheximide. The levels of newly synthesized MGSA-protein could not be measured due to the presence of exogenously administered growth factor and the lack of a good immunoprecipitating antibody. These experiments show that MGSA can increase the levels of its own mRNA by a mechanism of autostimulation.

We also examined whether cells other than the melanoma cell line Hs294T, synthesize MGSA. Polyadenylated RNA was prepared from various other cell lines and from several tumor biopsies. Northern hybridization showed that MGSA mRNA can be found in the BD melanoma cell line (not shown), the renal carcinoma cell line CAK-1-68, and the PC3 prostatic carcinoma cell line (Figure 6). Human foreskin fibroblasts FS4 gave also a strongly hybridizing band, but IM-9 lymphocytes were MGSA mRNA negative (not shown). Analysis of human tumor biopsies showed that MGSA mRNA was present in several squamous cell carcinomas and adenocarcinomas (Figure 6). The level of MGSA secretion by the cells could not be measured. Since we have found expression of the MGSA gene in a variety of cell types, it is likely that many tumor cells synthesize this growth factor. MGSA expression is therefore not restricted to the melanoma cells from which it was originally isolated.

Chromosomal location of the MGSA gene

A 730 bp fragment of the MGSA cDNA was used as probe in genomic Southern hybridizations. Two strongly hybridizing *Eco*RI fragments of 4.7 and 3.25 kb, and a much weaker fragment of 8.7 kb were present in human DNA (Figure 7, lane 3). These fragments were easily distinguishable from the single weakly hybridizing band in Chinese hamster DNA (Figure 7, lane 1). No distinct restriction fragments were seen in mouse (Figure 7, lane 2) and rat DNA (not shown). A relatively high background was present in the rodent DNAs and in all Chinese hamster × human hybrid DNAs, but not in human DNA suggesting that the hybridization probe may share sequence similarity to a rodent repetitive sequence.

Of the 16 Chinese hamster × human hybrids analyzed, five were positive for all three human *Eco*RI fragments; all others had only the hybridizing Chinese hamster fragment. This result is consistent with a single chromosomal site for the MGSA gene. Correlation of the human MGSA restriction fragments with the presence of human chromosomes in the 16 cell hybrids revealed perfect concordance with chromosome 4: All other chromosomes were ruled out by three or more discordant hybrids (Table II). For regional mapping, Southern analysis was carried out on four hybrids containing parts of human chromosome 4. Of these four hybrids, only the hybrid containing the entire long arm of chromosome 4 was positive for the human MGSA fragments. These results place the MGSA gene on the proximal long arm, in region cen→q21, of chromosome 4 (Figure 8, left).

The chromosome assignment of the human MGSA gene was confirmed by *in situ* hybridization of the ^{3}H-labelled MGSA cDNA to metaphase chromosomes from a normal individual. In 30 metaphase cells selected for the presence of silver grains on chromosomes 4 and/or 5, 32 grains were on chromosome 4 and 9 were on chromosome 5. The grains were non-randomly distributed with a peak at bands 4q13→q21 (Figure 8). These results confirm the localization determined by Southern blotting of somatic cell hybrid DNA

Table II. Correlation of human MGSA sequences wtih human chromosomes in rodent × human somatic cell hybrids

Hybridization/chromosome	Human chromosome																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
+/+	2	1	3	4	0	4	2	2	2	1	1	3	3	4	4	3	0	3	4	1	4	4	2
−/−	8	9	4	8	7	5	9	6	8	10	5	8	6	3	6	6	7	4	7	7	4	5	3
+/−	3	3	2	0	5	1	3	2	2	4	4	2	2	1	1	2	5	2	1	4	1	0	0
−/+	3	2	5	0	3	5	0	5	2	1	3	3	5	6	5	3	3	5	4	4	6	6	3
Discordant hybrids	6	5	7	0	8	6	3	7	4	5	7	5	7	7	6	5	8	7	5	8	7	6	3
Informative hybrids	16	15	14	12	15	15	14	15	14	16	13	16	16	14	16	14	15	14	16	16	15	15	8

The numbers of hybrids that are concordant (+/+ or −/−) and discordant (+/− or −/+) with the human MGSA sequence are given for each chromosome. Hybrids in which a particular chromosome was structurally rearranged or present in fewer than 10% of cells were excluded.

and narrow down the site of the MGSA gene to band 4q13 or the proximal part of band 4q21 (Figure 8).

Discussion

We have determined the N-terminal amino acid sequence of a 13 and a 16 kd species of MGSA and have isolated and characterized cDNAs for the MGSA precursor polypeptide. The 1050 bp long cDNA is close to full size, since the mRNA is ~1.1−1.2 kb long as judged by Northern hybridization. It contains a relatively long 3′ untranslated sequence with several AT rich sequences which conform to the consensus sequence ATTTA. Such sequences are found in the 3′ untranslated regions of many inflammatory mediators and have been shown to be involved in the selective degradation of transiently expressed mRNAs (Shaw and Kamen, 1986). The deduced amino acid sequence is 107 residues long including the presumed N-terminal signal sequence. Comparison with the N-terminal protein sequence indicates that the mature MGSA polypeptides are generated by cleavage following the Gly-residue at position 34 and are therefore maximally 73 amino acids long. This predicted mol. wt value of 9.3 kd is lower than the estimations based on denaturing gel electrophoresis. Such anomalies have also been seen with several other growth factors (Stroobant *et al.*, 1985; Betsholz *et al.*, 1986; Bringman *et al.*, 1987; Wong *et al.*, 1987). It is as yet unclear what causes the difference between the 13 and 16 kd species. C-terminal cleavage, e.g. at the Lys−Lys (positions 94−95 in Figure 2), or other post-translational modifications such as *O*-glycosylation are possibilities. However, it cannot be excluded that the 16 kd species represents a modified and N-terminally blocked form of MGSA and that the directly determined N-terminal sequence was derived from a small amount of 13 kd MGSA that was present in the 16 kd fraction. The mature MGSA polypeptides contain four cysteine residues. Comparison with the homologous sequence of β-thromboglobulin for which disulfide-bridge formation has been determined (Begg *et al.*, 1978), suggests the presence of two disulfide bridges between the first and third, and the second and fourth cysteines.

While this work was underway, Anisowicz *et al.* (1987) reported the isolation of a cDNA, that they named *gro* and that is nearly identical in sequence to our MGSA sequence. They found that the corresponding mRNA is transiently induced in normal fibroblasts following serum stimulation and that there is no such tight regulation of expression in transformed hamster fibroblast cells. Expression of this gene in non-transformed hamster fibroblasts did not result in any

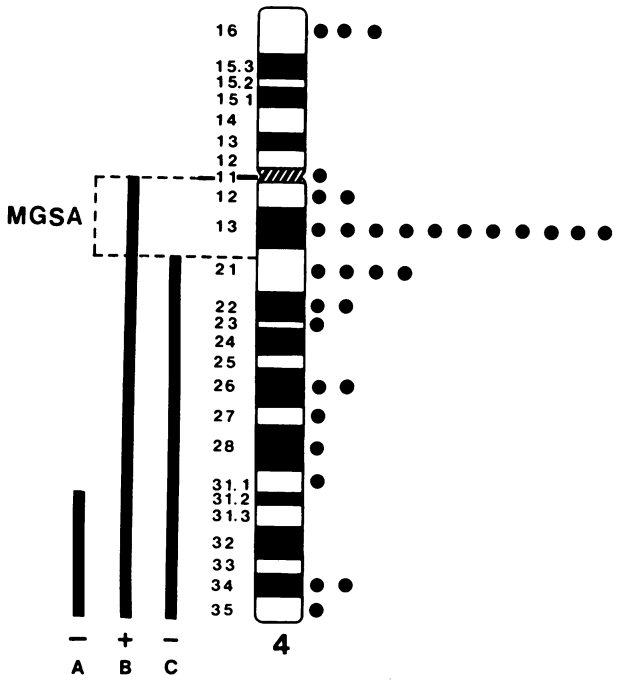

Fig. 8. Regional localization of MGSA on chromosome 4 by somatic cell hybrid analysis (**left**) and *in situ* hybridization (**right**). Vertical bars A, B and C represent regions of chromosome 4 retained in different hybrid cell lines. Only the hybrid with region B was positive (+) for human MGSA restriction fragments. Based on the combined results we map the *MGSA* locus to bands 4q13 to proximal 4q21.

detectable growth related differences, nor in the induction of hyaluronic acid or plasminogen activator synthesis (Anisowicz *et al.*, 1987). These latter activities are exerted by the structurally related CTAP-III on synovial fibroblasts (Castor *et al.*, 1983). Anisowicz *et al.* (1987) were therefore unable to assign a biological function to the *gro* gene product. In contrast, we have previously established that this gene product, called MGSA, is a growth factor for melanoma cells and for several immortalized fibroblast cell lines and is active at the nanogram level (0.05−10 ng/ml) (Richmond *et al.*, 1982, 1983; Richmond and Thomas, 1986). We have also shown that expression of the MGSA cDNA in mammalian cells results in the secretion of a factor with mitogenic activity for melanoma cells. It is possible that the receptors for MGSA were not present on the hamster fibroblasts used by Anisowicz *et al.* (1987) or that these cells were not responsive under the conditions tested.

The Northern hybridization data presented here and

previous immunohistochemical data (Richmond and Thomas, 1988) indicate that MGSA is expressed not only in melanoma cells, but also in various other cell types, including renal and prostate carcinoma as well as squamous cell and adenocarcinomas. The Northern hybridization data published by Anisowicz *et al.* (1987) are in agreement with this conclusion. The immunohistochemical studies detected MGSA in metastatic small cell lung carcinoma, prostatic carcinoma, mesangial proliferative glomerular nephritis, sarcoidosis tissue, non-malignant nevus tissue and in the epidermis of the skin (Richmond and Thomas, 1988). Therefore it appears that MGSA or MGSA-like polypeptides are secreted by a variety of normal and tumor cells and may play a role in the proliferation of these tissues.

Our results show that the levels of MGSA mRNA are strikingly elevated following treatment of the melanoma cells with MGSA. Since these cells are MGSA responsive, this result implies the existence of a positive autocrine signal. Zullo *et al.* (1985) have implicated the existence of a negative feedback loop, linked to growth factor induced mitogenesis and executed by growth inhibitory proteins. These authors have shown that addition of PDGF to rodent fibroblasts induces the expression of interferon-β mRNA. Such a role could also be fulfilled by TGF-β, a potent growth inhibitor for many cells (Tucker *et al.*, 1984; Sporn *et al.*, 1987). In contrast to such a negative feedback loop, there is now also evidence for an auto-stimulatory feedback mechanism. This is illustrated here by the induction of MGSA mRNA expression by MGSA in melanoma cells. Also, PDGF has been shown to induce the expression of PDGF A-chain mRNA in diploid fibroblasts (Paulsson *et al.*, 1987), while TGF-α and the structurally related epidermal growth factor (EGF) induce higher expression of TGF-α in keratinocytes (Coffey *et al.*, 1987). These finding suggest that several positive autocrine feedback mechanisms may exist in various cell types and that such auto-induction mechanisms likely exist for several other growth factors. Growth factor induced autostimulation may serve as an amplifier of the mitogenic response under certain conditions.

We have mapped the MGSA gene to chromosome 4(q13→q21). This same region contains the genes for platelet factor 4 (Griffin *et al.*, 1987) and for the interferon-γ inducible factor γIP-10 (Luster *et al.*, 1987). As shown in Figure 3, MGSA is structurally related to platelet factor 4 and γIP-10 and to various other polypeptide sequences. The localization of the genes for these other polypeptides has not been reported yet, but the co-localization of the MGSA, platelet factor 4 and γIP-10 genes suggests that they may be clustered in the same chromosome segment. It is also remarkable that the c-*kit* gene has been assigned to the same region as the MGSA gene (Yarden *et al.*, 1987). It has recently been found that the c-*kit* proto-oncogene encodes a new cell surface receptor. This receptor has a tyrosine-specific autophosphorylation activity and is structurally and functionally related to various other receptors for growth factors. However, the ligand for the c-*kit* receptor has not yet been identified, but is likely to be a growth factor (Yarden *et al.*, 1987). Genes for ligands and their corresponding receptors are usually found on different chromosomes, but few have been mapped to similar chromosomal regions (Francke *et al.*, 1984; Huebner *et al.*, 1985; Nienhuis *et al.*, 1985; Pettenati *et al.*, 1987). It will, therefore, be important to determine if the growth factor MGSA constitutes the ligand for the c-*kit* receptor. This region also contains the gene for 'piebald trait', an inherited disorder that results in abnormal skin pigmentation with patchy hypo- and hyperpigmentation. This disorder is due to a genetic deficiency related to melanoblast migration and differentiation (Hoo *et al.*, 1986). In the mouse, the region of chromosome 5, that is known to be homologous to the proximal long arm of the human chromosome 4 (Searle *et al.*, 1987) contains a locus *W* that also gives rise to pigmentary changes when mutated (Gordon, 1977). It is possible that MGSA from the gene in this chromosome 4 locus has an effect not only on melanoma cells, but also on melanocyte differentiation or migration and in this way may be involved in the establishment of the 'piebald trait'.

MGSA shows unambiguous sequence similarity with several other polypeptides. Of these, the only other one for which there is evidence of growth factor activity is CTAP-III, a mitogen for synovial fibroblasts which is released by platelets (Castor *et al.*, 1983). CTAP-III is a processed form of PBP (Holt *et al.*, 1986). Another homologous polypeptide, platelet factor 4 (Deuel *et al.*, 1977), is also stored in platelets but it is not known whether this factor can serve as a mitogen. Furthermore, MGSA is structurally related to the deduced protein sequences for three other cDNAs. One of them, γIP-10 is synthesized following stimulation of several cell types with interferon-γ (Luster *et al.*, 1985), while 3-10C is induced in leukocytes by mitogenic treatment with Staphylococcal enterotoxin A (Schmid and Weissman, 1987). The third cDNA, designated 9E3 (Sugano *et al.*, 1987) or PCEF-4 (Bedard *et al.*, 1987), is induced when chicken embryo fibroblasts undergo transformation by Rous sarcoma virus. No function has as yet been assigned to the gene products of these three cDNAs. However, since both MGSA and CTAP-III can serve as mitogens for certain cell populations, it becomes now more likely that these factors with unambiguous sequence similarity and conservation of all four cysteines (Figure 3) constitute a new family of growth factors. Thus, the 3-10C polypeptide (Schmid and Weissmann, 1987) could possibly be involved in the mitogenic stimulation of leukocytes following Staphylococcal enterotoxin A treatment. Similarly, the 9E3 (or pCEF-4) (Sugano *et al.*, 1987; Bedard *et al.*, 1987) protein could be a growth factor that is involved in an autocrine growth stimulation following Rous sarcoma virus transformation. A mitogenic role of the interferon-γ induced polypeptide γIP-10 may be more doubtful, since interferon-γ exerts antiproliferative activities in several cell systems. However, Brinckerhoff and Guyre (1987) have documented an interferon-γ induced increase in proliferation of synovial fibroblasts. It is thus possible that these structurally related factors are members of a family of growth factors, which could play a role in autocrine or paracrine growth stimulation of various cell types. Extensive studies should now be undertaken in order to characterize the biological roles of MGSA and of the other homologous peptides.

Materials and methods

Determination of the N-terminal amino acid sequence of MGSA

MGSA was purified by Biogel P-30 gel filtration and heparin-sepharose and the 13 and 16 kd species were then separated by HPLC (Thomas and Richmond, 1988). The 13 and the 16 kd MGSA peptides were subjected to Edman degradation in an Applied Biosystems 670A protein sequencer

equipped with an Applied Biosystems 120A phenyl thiohydantoin analyzer and a Hewlett-Packard 3393A integrator as described (Fisher and Spiess, 1987). A total of 10.5 μg (0.8 nmol) of the 13 kd species was used for two N-terminal analyses and the recovery was 38–75% for the first two cycles. A total of 7.2 μg (0.45 nmol) of the 16 kd fraction was subjected to analysis but the recovery was less than 5% for the third cycle.

mRNA isolation and cDNA isolation

The cell line Hs294T, derived from a human metastatic malignant melanoma (Creasy *et al.*, 1979) was grown to confluence in Ham's F-10 medium, supplemented with 10% fetal calf serum. Forty-eight hours prior to the harvest, the cells were incubated in serum-free F-10 medium. Total RNA was extracted as described (Rosenthal *et al.*, 1986) except that heparin was not included, and the polyadenylated mRNA fraction was isolated by oligo(dT)–cellulose chromatography (Aviv and Leder, 1972). The cDNA synthesis was carried out using a reagents kit obtained from Amersham. The first strand synthesis (Huynh *et al.*, 1984) was done using avian myeloblastosis virus reverse transcriptase and oligo dT12-18 as primer and was followed by RNase H and DNA polymerase I for second strand synthesis (Gubler and Hoffman, 1983). The asymmetric synthetic *Eco*RI linkers 5′-AATTCTCGAGCTCACCTGC and 5′-GAGCTCGAGTGGACG were ligated to the double stranded cDNA. The cDNA library was subsequently constructed in λgt10 as described (Huynh *et al.*, 1981). The other two cDNA libraries, one derived from placenta mRNA and the other from the melanoma cell line 3728 were obtained from Dr Axel Ullrich.

Three long oligonucleotides that correspond to the N-terminal sequence of the 13 kd species of MGSA (Figure 1) were used for the isolation of a MGSA cDNA. The 45mer 5′-GCCTCCGTGGCCACCGAGCTGAGG-TGCCAGTGCCTGCAGACCCTG, corresponding to amino acids 1–15, and the 42mer 5′-CTGCAGACCCTGCAGGGCATCCACCCCAAGAAC-ATCCAGTCC covering amino acids 12–25, were designed on the basis of the codon bias observed in human mRNAs (Grantham *et al.*, 1981). The third oligonucleotide, the 63mer 5′-ACCCTGCAGGGAATTCACCCC-AAGAACATCCAAAGTGTGAACGTGAAGTCCCCCGGTCCCCAC corresponds to amino acids 14–34 (compare with Figure 1) and was designed on the basis of a preliminary cDNA sequence for *gro*, communicated by Dr Sager (Anisowicz *et al.*, 1987). These oligonucleotides were 5′ labeled with [^{32}P-γ]ATP and polynucleotide kinase as described (Maniatis *et al.*, 1982) and were used as hybridization probes in low stringency hybridization condition (Yarden *et al.*, 1987). The cDNA libraries from placenta mRNA and from the melanoma cell line 3728 were screened by hybridization with the two 42mers separately. The cDNAs isolated with either probe were then hybridized to the 63mer. This led to the isolation of an incomplete MGSA cDNA from the placenta library. This cDNA was labeled using the random primer labeling technique (Feinberg and Vogelstein, 1983) and used to screen about 1.5 × 10^6 λgt10 plaques of the Hs294T cDNA library under high stringency conditions (Derynck *et al.*, 1985). About 12 cDNAs, most of which were about 1050 bp long, were isolated.

Nucleotide sequence analysis was carried out by subcloning into M13 vectors (Messing *et al.*, 1987) followed by primed DNA synthesis on single stranded DNA templates in the presence of dideoxynucleotide triphosphates (Smith, 1980). The cDNA sequence shown in Figure 3 was based on the analysis of three cDNAs.

Expression of the MGSA cDNA

The expression vector pMGSA 5-2/3 (Figure 4) was constructed by ligation of the following fragments. Starting from the *Cla*I site and proceeding clockwise, there is first the MGSA cDNA containing the entire coding sequence, followed by the proximal part of the untranslated region up to the *Pst*I site (position 424 in Figure 2). This is ligated onto the *Hpa*I–*Bam*HI fragment that contains the polyadenylation site of the SV40 late transcript (Fiers *et al.*, 1978). This segment that has not retained its *Hpa*I and *Bam*HI sites is followed by a segment of SV40 DNA (originally a *Kpn*I–*Hin*dIII fragment) that comprises the replication origin and early and late promoters (Fiers *et al.*, 1978). This segment is in turn followed by a segment that corresponds to the *Sal*I–*Eco*RI fragment of pML-1 (Lusky and Botchan, 1981), comprising the pBR322 origin of replication and the β-lactamase gene. Finally, the long terminal repeat of Rous sarcoma virus located on a 600 bp DNA segment (Gorman *et al.*, 1982) serves as the promoter for the MGSA cDNA.

The MGSA expression plasmid and the plasmid pRSV Neo (Gorman *et al.*, 1983) were transfected into kidney cells as described (Eaton *et al.*, 1986). Samples were obtained after culturing the transfected cells for 48 h in serum-free medium. These samples were diluted before assay for stimulation of DNA synthesis in the Hs294T melanoma cells (Richmond and Thomas, 1986, 1988). Assays were done in triplicate.

Northern blot hybridization analysis

Hs294T melanoma cells were treated in four different ways. One Hs294T culture was incubated for 72 h in serum free medium, while the second culture received the same treatment except that 10 μg/ml of cycloheximide were present during the last 4 h. The third melanoma culture was incubated in serum free medium for 24 h and then treated with 3 ng/ml of the 13 kd species of MGSA for 48 h. The fourth culture received the same treatment as the third culture except that cycloheximide was present at 10 μg/ml for the last 4 h. Total cytoplasmic RNA was prepared using the guanidinium–CsCl method (Maniatis *et al.*, 1982).

RNA from all other cell lines and tumor biopsies were isolated by the guanidinium thiocyanate–LiCl method (Cathala *et al.*, 1983). The cell lines and biopsies were described previously (Derynck *et al.*, 1987).

Three micrograms of poly(A)$^+$ mRNA obtained after chromatography on oligo(dT)–cellulose (Aviv and Leder, 1972) were denatured at 65°C in the presence of formamide and formaldehyde and then subjected to electrophoresis on a 1.2% agarose gel in the presence of formaldehyde (Maniatis *et al.*, 1982) and thereafter blotted to nitrocellulose filter. Hybridizations were performed under high stringency conditions. The 0.7 kb *Eco*RI fragment of the MGSA cDNA insert isolated from the placenta cDNA library, was used as a hybridization probe.

Chromosome localization methodology

For the mapping of the MGSA gene on human chromosomes, 16 somatic cell hybrid clones derived from seven different series of Chinese hamster × human hybrids were analyzed by Southern blotting. The origin and human chromosome content of these hybrids have been summarized recently (Yang-Feng *et al.*, 1986). In addition, four hybrids containing parts of the human chromosome 4 were used for regional localization. In two of them, spontaneous translocation of 4 cen→qter or of 4q21→qter, respectively, to a Chinese hamster chromosome had taken place during growth in culture. Trypsin-Giemsa banded and Giemsa-11 stained examples of the rearranged chromosomes have been published (Brissenden *et al.*, 1984). Two other hybrids were derived from human donor cells carrying a balanced t(X;4) translocation; both of these contained region 4q31.2→qter translocated to the X chromosome in the absence of a normal chromosome 4. The hybridization probe used for Southern blotting was a 700 bp *Eco*RI fragment of the MGSA cDNA. This fragment corresponds to a distal segment of the MGSA coding sequence and the 3′ untranslated region. This cDNA fragment was labelled with [^{32}P]dCTP (Feinberg and Vogelstein, 1983).

In situ hybridization to metaphase chromosomes from a normal individual was done following published procedures (Harper and Saunders, 1981; Yang-Feng *et al.*, 1985). The pUC118 plasmid containing the 900 bp MGSA cDNA *Eco*RI fragment was labelled with tritiated nucleotides by nick-translation (Maniatis *et al.*, 1982).

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