The EMBO Journal vol.9 no.4 pp.1087 – 1096, 1990

In vivo transformation of factor-dependent hemopoietic cells: role of intracisternal A-particle transposition for growth factor gene activation

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Communicated by T.Graf

Cells of the granulocyte-macrophage colony stimulating factor (GM-CSF) or multi-lineage colony stimulating factor (Multi-CSF) dependent line FDC-P1 undergo leukemic transformation after injection into irradiated DBA/2 mice. About one third of factor-independent FDC-P1 variants isolated from leukemic animals express GM-CSF or Multi-CSF, assessed either by bioassay or by sensitive RNA detection using the polymerase chain reaction. All of the GM-CSF-secreting lines studied had a rearrangement in one allele of the GM-CSF gene, three of four Multi-CSF-secreting lines had Multi-CSF gene rearrangements, while factor-independent lines lacking evidence of growth factor production had no demonstrable CSF gene alterations. All rearrangements were characterized by insertions of novel DNA in the 5′-flanking regions of the CSF genes. The inserted segments of DNA varied in size between 0.35 and 6.5 kb and displayed restriction enzyme cleavage maps reminiscent of intracisternal A-particle (IAP) genomes. This was confirmed in two cases by molecular cloning and nucleotide sequence analysis. In these instances, the insertion consisted of solitary IAP long terminal repeats. The transformation system described provides a model for the study of IAP transpositions and their effects on gene activation.

Key words: autocrine growth stimulation/CSF genes/IAP/polymerase chain reaction

Introduction

Factor-dependent hemopoietic cells lines (Dexter *et al.*, 1980; Greenberger *et al.*, 1983) are valuable tools for the study of leukemogenesis. Like normal hemopoietic progenitor cells, such lines exhibit an absolute dependency on stimulation by the appropriate growth factors (the colony stimulating factors, or CSFs; Metcalf, 1984) for survival and proliferation *in vitro*. However, terminal differentiation and eventual cell death, which invariably accompany the proliferation of normal hemopoietic cells, are replaced by a capacity for unlimited self-renewal. Despite these properties, factor-dependent cell lines are generally not tumorigenic *in vivo*, possibly because the tissue CSF concentrations are not sufficient to stimulate proliferation, or because inhibitory mechanisms effectively antagonize the stimulatory signals. Since the cells of factor-dependent lines can be viewed as immortalized equivalents of normal cells, extensive efforts have been made to define conditions that allow their further progression to malignancy. These studies have almost exclusively been confined to *in vitro* experiments, either focussing on the effects of artificially introduced genes on cell proliferation (Adkins *et al.*, 1984; Cook *et al.*, 1985; Lang *et al.*, 1985; Rapp *et al.*, 1985; Hapel *et al.*, 1986; Metcalf *et al.*, 1987; Wheeler *et al.*, 1987; Wong *et al.*, 1987; Hariharan *et al.*, 1988; Chang *et al.*, 1989; Nair *et al.*, 1989) or alternatively, analyzing the behavior of spontaneous *in vitro* mutants (Schrader and Crapper, 1983; Naparstek *et al.*, 1986; Boswell *et al.*, 1987; Stocking *et al.*, 1988; Leslie and Schrader, 1989). While such approaches allow an assessment of the importance of particular genes in the transformation process, they do not necessarily provide information on the mechanisms involved in the development of leukemia *in vivo*.

As an alternative to the outlined approaches, we have recently described an *in vivo* transformation system for FDC-P1 cells, a non-tumorigenic murine hemopoietic cell line whose proliferation *in vitro* depends on stimulation by either granulocyte-macrophage colony stimulating factor (GM-CSF) or multi-lineage colony stimulating factor (Multi-CSF, also called interleukin 3) (Dexter *et al.*, 1980). Following intravenous injection with these cells, sub-lethally irradiated DBA/2 mice ultimately develop transplantable leukemias derived by transformation of the injected cells (Dührsen and Metcalf, 1988). The role of irradiation is not yet fully understood, but an important component appears to be a dramatic acceleration of the proliferation of untransformed FDC-P1 cells in the preleukemic phase (Dührsen and Metcalf, 1989a,b). The first leukemic transformants become detectable 6–10 weeks after injection (Dührsen and Metcalf, 1989a); these subsequently suppress normal hemopoiesis, invade extra-hemopoietic tissues and eventually lead to the death of the animal (Dührsen and Metcalf, 1988). In the leukemic cell populations of ~80% of the animals, factor-independent FDC-P1 variants are detectable, which frequently give rise to continuous cell lines (Dührsen, 1988; Dührsen and Metcalf, 1988). While for most such lines the mechanisms underlying growth autonomy remain unknown, about a third produce either GM-CSF or Multi-CSF (Dührsen, 1988). Since retrovirus-mediated expression of CSF genes induces factor independence and tumorigenicity in FDC-P1 cells, probably via autocrine stimulation (Lang *et al.*, 1985; Hapel *et al.*, 1986; Laker *et al.*, 1987), it is tempting to speculate that activation of CSF genes is also a crucial transforming event in the *in vivo* mutants. It appears noteworthy however, that in our *in vivo* model CSF gene activation appears to be less a frequent event than in *in vitro* transformation systems for factor-dependent cell lines, in which acquisition of growth autonomy is almost invariably associated with autocrine production of CSFs (Stocking *et al.*, 1988). This difference may be related to different

Table I. Production of CSFs by factor-independent FDC-P1 variants isolated from leukemic DBA/2 mice

Mouse	Number of cell lines isolated with autogenous production of:		
	GM-CSF	Multi-CSF	No CSFs
11	11	0	0
E	3	0	0
12	1	3	0
5	0	6	0
6	0	1	18
2	0	1	6

[a]Mice 11, 12, 6 and 2 received 3.5 Gy irradiation prior to injection of 10^6 FDC-P1 cells, mouse E recieved 1 Gy and mouse 5 was not irradiated (Dührsen, 1988).

properties of the specific cell lines used or may reflect peculiarities of the conditions of transformation *in vivo* and *in vitro*.

The present investigations were undertaken to elucidate the molecular mechanisms underlying the activation of CSF genes in the *in vivo*-derived leukemic FDC-P1 variants. They revealed that CSF gene activation is almost invariably associated with insertion in the proximity of the CSF genes of genetic elements displaying the hallmarks of intracisternal A-particles IAPs.

Results

Expression of CSF genes by factor-independent FDC-P1 variants

In order to ascertain whether autocrine growth factor production might be involved in the leukemic transformation of FDC-P1 cells *in vivo*, cloned FDC-P1 variant cell lines derived from lymph nodes of 17 leukemic mice were tested for growth autonomy and autogenous CSF production (Dührsen, 1988). Factor-independent FDC-P1 variants secreting either GM-CSF or Multi-CSF were isolated from six of these leukemic animals (Table I), whereas the cell lines from 11 mice failed to provide unequivocal evidence of CSF production. Table I shows that in two of the mice from which CSF-secreting leukemic cells were derived, CSF production was limited to a minority of the cell lines isolated, and in one case lines differing in their capacity to produce either GM-CSF or Multi-CSF were detected within the same leukemic cell population. The overall frequency of GM-CSF or Multi-CSF production by leukemic FDC-P1 variants was ~30%, both with respect to the number of mice studied (6/17) and the total number of factor-independent cell lines analyzed (26/79).

Initially CSF production was demonstrated by bioassay of conditioned media, using the factor-dependent indicator cell lines FDC-P1, which responds to GM-CSF or Multi-CSF, and 32D clone 3, which responds only to Multi-CSF. The identity of the factor expressed by the variant lines was confirmed in two approaches: neutralization assays using specific antibodies against GM-CSF or Multi-CSF and detection of specific CSF transcripts. Figure 1 shows representative neutralization experiments for the conditioned medium from one cell line (E/1) with exclusive FDC-P1-stimulating activity (suggestive of GM-CSF secretion), and the media from two lines (12/3 and 2/7) able to stimulate both FDC-P1 and 32D cells (compatible with secretion of

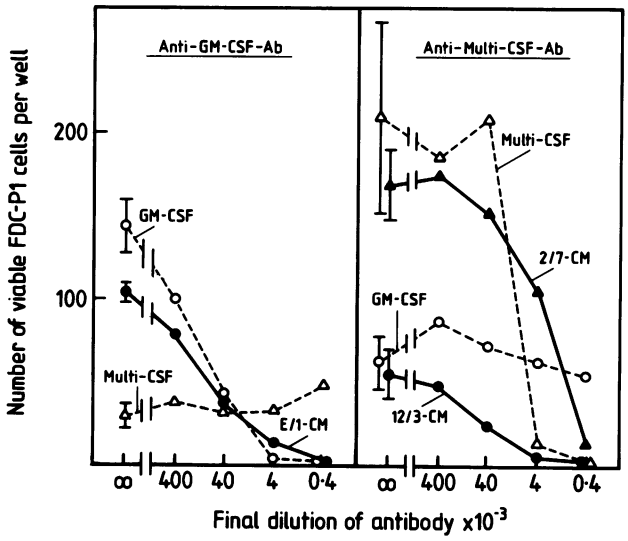

Fig. 1. Neutralization of active conditioned media from factor-independent FDC-P1 variants by specific CSF antibodies. **Left panel.** Ability of anti-GM-CSF antibody to inhibit stimulation of FDC-P1 cells by 8 U/ml purified GM-CSF or conditioned medium (CM) from line E/1. Failure of the antibody to block stimulation by 4 U/ml purified Multi-CSF. **Right panel.** Ability of anti-Multi-CSF-antibody to inhibit stimulation by 8 U/ml Multi-CSF and CM from lines 12/3 or 2/7, respectively. Failure to block GM-CSF-stimulated FDC-P1 survival (4 U/ml). CM from line 12/3 was prediluted to give ~50% maximal stimulation, CM from line E/1 was undiluted, and CM from line 2/7 was 25-fold concentrated.

Multi-CSF). Specific inhibition by CSF antibodies confirmed the presence of GM-CSF in conditioned medium from line E/1 and Multi-CSF in conditioned medium from lines 12/3 and 2/7.

Bioassays of cell lysates gave qualitatively similar results to those obtained with conditioned media (Table II). Interestingly however, the extractable intracellular or cell-associated CSF concentration was significantly higher (up to 25 000 times) on a per cell-volume basis than the concentration in the culture medium. Although cultures of untransformed FDC-P1 cells stimulated by exogenous GM-CSF also showed higher intracellular or cell-associated than extracellular CSF concentrations (presumably resulting from binding or internalization of exogenous CSF), in this case only a 25-fold difference was observed. Whether the higher intracellular CSF concentrations in the factor-independent lines are due to preferential binding of factor to the producer cell, or reflect inefficiency of secretion, is unclear.

Using conventional Northern blots, we failed to detect GM-CSF or Multi-CSF mRNA in the majority of lines tested (data not shown), probably because the amounts of CSF produced were generally very small (Table II). We therefore used the polymerase chain reaction (PCR), as a very sensitive means of specific RNA detection. PCR-based RNA analysis confirmed the pattern of CSF gene expression obtained by bioassay and also revealed that a number of Multi-CSF-producing lines simultaneously expressed low levels of GM-CSF RNA (Figure 2). The converse, however, was not observed.

From each of the six mice that had given rise to GM-CSF or Multi-CSF-producing leukemic cells, two to six factor-independent cell lines were selected for further molecular analysis and compared with untransformed FDC-P1 cells and factor-independent control lines lacking evidence of CSF

Table II. Summary of CSF production, mRNA expression and gene rearrangements in factor-independent FDC-P1 variants

Designation of cell line[a]	Biological CSF activity (U/ml)		PCR-based mRNA detection		Gene rearrangement	
	Conditioned media[b]	Lysates[c]	GM-CSF	Multi-CSF	GM-CSF	Multi-CSF
GM-CSF producing lines						
11/2, 11/11	3–9	10 000–150 000	+++	–	+(C)[d]	–
E/1, E/2	0–1	1000–10 000	+++	–	+(B)	–
12/10	0[e]	20 000–80 000	+++	–	+(A)	–
Multi-CSF producing lines						
12/1, 12/3, 12/7	300–2000	5000–150 000	+	+++	–	+(F)
5/1 - 5/6	6–10	10 000–150 000	+[f]	+++	–	+(D)
6/11	4	540–2600	–	+++	–	+(E)
2/7	<1[g]	0	–	+	–	–
Other lines						
6/1 - 6/6	0[h]	0	–	–	–	–
2/8	0	0	–	–	–	–
3/3, 3/10[i]	0	0	–	–	–	–
4/12, 4/15[i]	0	0	–	–	–	–
Control FDC-P1	400[j]	10 000[j]	–	–	–	–

[a]First number or letter designates leukemic animal (cf. Table I), second number individual clones isolated from these animals.
[b]Media conditioned by 5×10^5 cells for 48 h.
[c]Calculated intracellular CSF concentrations (cf. Materials and methods).
[d]Letters refer to maps in Figure 5.
[e]Up to 6 U/ml in overgrown cultures (cf. Materials and methods).
[f]Variable expression, cf. Figure 2.
[g]Only demonstrable in concentrated conditioned media.
[h]For some lines, trace amounts of FDC-P1 and 32D cell-stimulatory activity in 25-fold concentrated media. No further characterization.
[i]Small amounts of FDC-P1 cell stimulating activity in 25-fold concentrated conditioned media.
[j]400 U/ml exogenous GM-CSF added to cultures.

production (isolated from two other mice, numbers 3 and 4 in Table II).

CSF gene rearrangements in CSF-producing FDC-P1 variants

In order to investigate whether CSF production was associated with alterations in the respective genes, Southern blot analysis was performed with genomic DNA from the cell lines listed in Table II, and a panel of 11 different restriction enzymes. The probe used for characterization of the GM-CSF locus was a GM-CSF cDNA and for the Multi-CSF locus, a 1.9 kb genomic *Hin*dIII fragment encompassing part of exon 1 and the 5′-flanking region (Figure 5). All GM-CSF producing lines (for example lines 11/11, E/1 and 12/10 in Figure 3) showed a rearrangement of one allele of the GM-CSF gene (Figure 3) and a germline configuration of the Multi-CSF gene (Figure 4), whereas all Multi-CSF-producing lines (except for line 2/7) showed a rearrangement of one allele of the Multi-CSF gene (Figure 4) without evidence of any GM-CSF gene mutation (Figure 3). The CSF genes in the factor-independent lines that lacked evidence of CSF production were in the germline configuration. The GM-CSF genes of different GM-CSF-producing lines originally isolated from the same leukemic animal showed identical abnormalities, suggesting that these lines were siblings. The same was true for the Multi-CSF-producing lines. Cell lines derived from different mice, however, differed in their restriction patterns, indicating independent genetic changes. Likewise, lines from the same animal which differed phenotypically, either in their ability to produce a CSF (e.g. line 6/11 compared with 6/1–5, see Figure 2) or in the type of CSF produced (e.g. line 12/3 compared with 12/10, see Figure 2), showed different genetic alterations. Thus, the DNA data confirmed the suggestion (Dührsen, 1988) that FDC-P1 derived leukemias

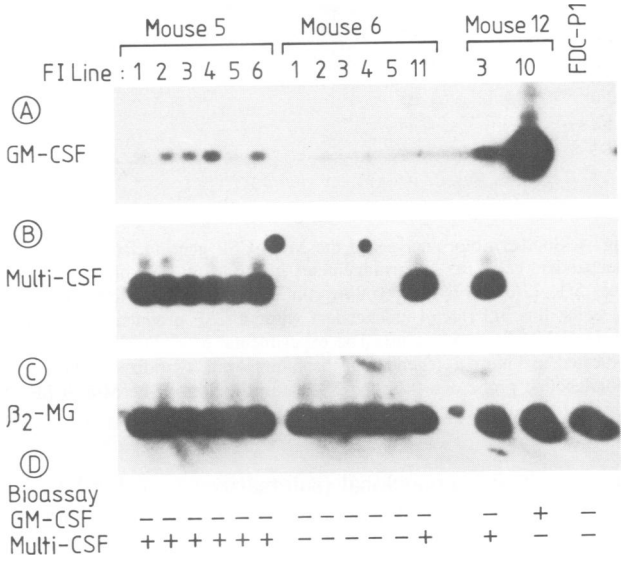

Fig. 2. PCR-based detection of mRNA from factor-independent FDC-P1 variants. Cytoplasmic RNA from factor-independent (FI) lines or control FDC-P1 cells was reverse transcribed into cDNA which was subjected to PCR amplification using specific oligonucleotides for GM-CSF (**A**), Multi-CSF (**B**), or, as a positive control, β_2-microglobulin (β_2-MG, **C**). The PCR products were electrophoresed in agarose gels, transferred to nitrocellulose filters and hybridized with radiolabeled cDNA probes specific for GM-CSF, Multi-CSF or β_2-MG, respectively. Panel **D** summarizes the results of CSF bioassays of the conditioned media from the cell lines (cf. Table II).

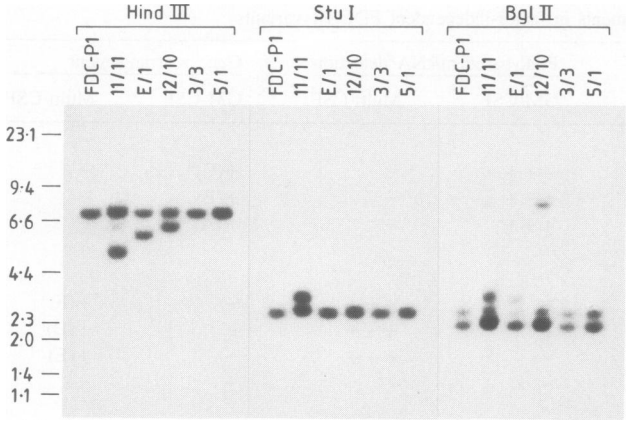

Fig. 3. Southern blot analysis of the GM-CSF gene in factor-independent cell lines. Rearrangements in the GM-CSF-producing lines 11/11, E/1 and 12/10; germline configuration in the original FDC-P1 cells, line 3/3 (factor-independent without CSF production) or line 5/1 (Multi-CSF production). Genomic DNA was digested with the restriction endonucleases indicated, electrophoresed and transferred to nitrocellulose filters which were hybridized with a radiolabeled GM-CSF cDNA probe. Lambda DNA digested with *Hin*dIII and ΦX174 DNA digested with *Hae*III were used as size markers.

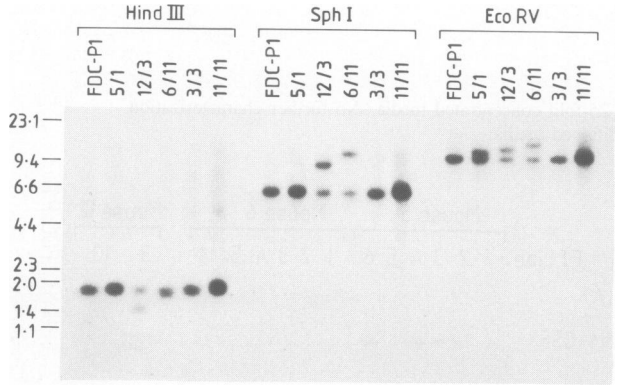

Fig. 4. Southern blot analysis of the Multi-CSF gene in factor-independent cell lines. Rearrangements in the Multi-CSF-producing lines 5/1, 12/3 and 6/11; germline configuration in the original FDC-P1 cells, line 3/3 (factor-independent without CSF production) or line 11/11 (GM-CSF production). The experimental procedures were as indicated in Figure 3 legend, but the filters were hybridized with a radiolabelled probe detecting the 5′-flanking region of the Multi-CSF gene (cf. Figure 5).

are not always monoclonal (summarized in Table II).

Construction of restriction maps for the rearranged alleles in three distinct GM-CSF-producing and three distinct Multi-CSF-producing lines suggested that the mutations were invariably related to insertions of DNA elements in the 5′-flanking region of the CSF genes (Figure 5). For lines 11/11 and E/1, inserts of 350 bp were apparent, which both carried a *Hin*dIII site (Figures 3 and 5), whereas for line 12/10 a much larger insert (5.5 kb) was evident (Figure 5). Two of the three Multi-CSF gene insertions appeared to have occurred within the region encompassed by the hybridization probe (Figure 5): a 4.2 kb insert in line 12/3 and a 6.5 kb insert in 6/11. The rearrangement in line 5/1 was not analyzed in detail, but appeared to have occurred at a considerable distance (~7 kb) from the Multi-CSF coding region. However, digestion of 5/1 DNA with restriction enzymes whose cleavage sites lay 5′ of the putative insertion locus invariably generated extra bands in addition to those obtained with FDC-P1 DNA, and the *Eco*RV and *Ssp*I digests both showed a 2.5 kb increase in fragment size, possibly reflecting the size of the inserted DNA element (Figures 4 and 5).

Cloning and sequencing of DNA inserts in the GM-CSF gene of lines 11/11 and E/1

The fact that the GM-CSF gene inserts in lines 11/11 and E/1 were mapped to lie within the region of known nucleotide sequence (Miyatake *et al.*, 1985a; Figure 5), enabled us to clone the respective rearranged regions after amplification by PCR technology. In line 11/11 the insertion was predicted to be between a *Stu*I site and a *Pst*I site (in the first exon), which were therefore used to define the position and sequence of the 5′ and 3′ PCR oligonucleotides (Figures 5 and 6C). For line E/1, the oligonucleotides used for PCR amplification of the rearranged region spanned a fragment between the beginning of the known nucleotide sequence and a sequence 520 bp further downstream (Figure 6C). The oligonucleotides were 25- to 27mers with homology to 17 bp of the respective GM-CSF gene sequence and overhanging *Eco*RI (5′ oligonucleotides) or *Bam*HI (3′ oligonucleotides) restriction sites respectively, which were added to facilitate subsequent ligation into cloning vectors.

Amplifcation by PCR of the rearranged regions was performed using different combinations of oligonucleotides and at several different annealing temperatures. While at annealing temperatures of 40°C and 50°C multiple non-specific bands were generated in addition to a main band corresponding to the unrearranged GM-CSF allele (e.g. Figure 6A), increasing the annealing temperature to 60°C resulted in virtual disappearance of the non-specific bands (note FDC-P1 DNA in Figure 6B). For lines 11/11 and E/1, however, a double band of the size expected for the rearranged GM-CSF allele (Figure 6B) was also seen. This doublet was only observed when suitable oligonucleotides were used (see Figure 6B) in combination with template DNA from lines 11/11 or E/1, respectively, and not with unrearranged FDC-P1 DNA. Furthermore, this doublet hybridized specifically with a GM-CSF probe on Southern transfer (not shown). Whilst it is unclear why PCR amplification of the rearranged allele consistently generated a doublet, nucleotide sequences of independent subclones derived from these fragments (below) did not differ in length, suggesting either an electrophoretic artefact or possibly accumulation of single-stranded DNA during the PCR reaction. The double bands generated at 60°C annealing temperature from 11/11 or E/1 template DNA, respectively, were recovered from the gel, digested with *Eco*RI and *Bam*HI and ligated into appropriate cloning vectors for sequencing.

Nucleotide sequence analysis revealed that in line 11/11 a fragment of 352 bp had been inserted between positions 980 and 981 of the published nucleotide sequence (Miyatake *et al.*, 1985a), that is, 126 nucleotides (nt) upstream from the TATA box (Figure 7A). The insertion was flanked by a 6 bp duplication of positions 975–980. The flanking GM-CSF gene sequence was identical to that published by Stanley *et al.* (1985) and differed from that of Miyatake *et al.* (1985a) by a substitution of T by AG in position 917. The rearrangement in line E/1 was due to a 366 bp insertion between positions 201 and 202 of the GM-CSF gene, that is, 905 nt upstream of the TATA box and 62 nt downstream

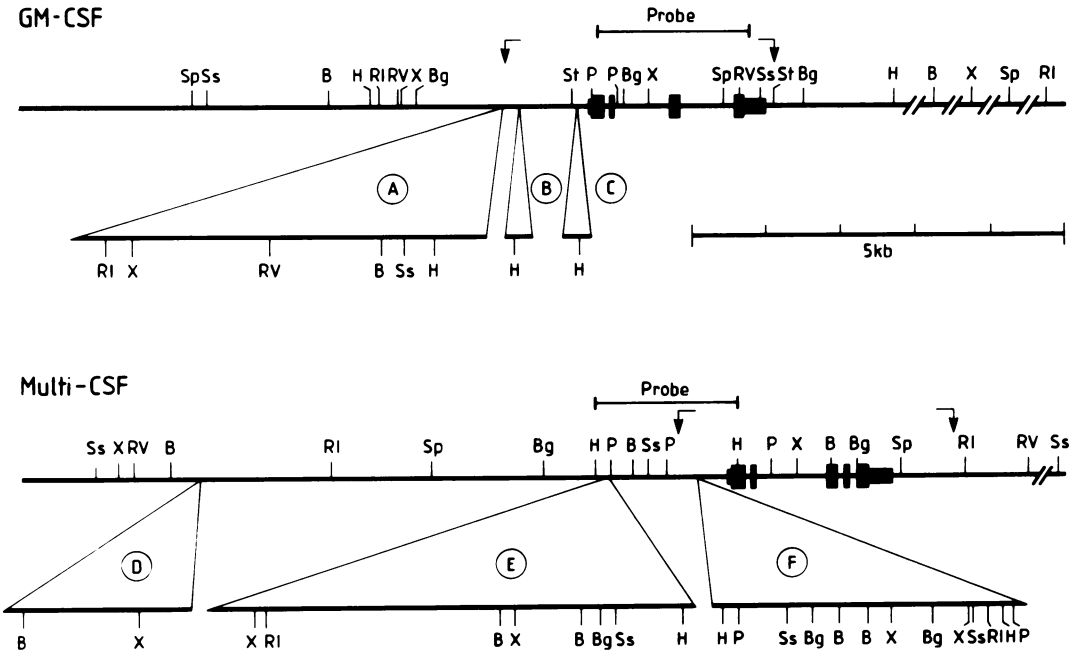

Fig. 5. Mapping of the rearrangements in the GM-CSF gene (**top**) and the Multi-CSF gene (**bottom**) of factor-independent FDC-P1 variants. Insertion A was found in line 12/10, B in E/1 and E/2, C in 11/2 and 11/11, D in 5/1-6, E in 6/11 and F in 12/1, 12/3 and 12/7 (see Table II for details of these cell lines). Exons are represented as black boxes. Arrows indicate the boundaries of the known nucleotide sequence of the GM-CSF gene (Miyatake *et al.*, 1985a; Stanley *et al.*, 1985) and the Multi-CSF gene (Miyatake *et al.*, 1985b; Campbell *et al.*, 1985). The hybridization probes used were a GM-CSF cDNA and a 1.9 kb genomic *Hin*dIII fragment detecting part of Multi-CSF exon 1 and the 5′-flanking region. Abbreviations for restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII ; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; Sp, *Sph*I, Ss, *Ssp*I; St, *Stu*I; X, *Xba*I. Not indicated (but consistent with our interpretations) are the results of *Dra*I digests.

from a GT repeat exhibiting homology to enhancer sequences (Miyatake *et al.*, 1985a) (Figure 7B). This insert was also flanked by a 6 bp target site duplication, affecting positions 196–201. The flanking GM-CSF gene sequence showed four deviations from the published sequence. These discrepancies were also found in unrearranged DNA from FDC-P1 cells cloned in a similar manner to that described for the rearranged alleles. Instead of the published 14 GT repeats in positions 112–139, three out of nine M13 clones sequenced showed 21, four 23, and two 24 GT repeats. Likewise, instead of a single TG in positions 149–150, all clones showed a TGTG repeat. The other differences were substitutions of T by C in positions 186 and 352, respectively.

The inserted sequences in lines 11/11 and E/1 were almost identical to each other (95% identity), and were highly homologous to published sequences of the long terminal repeats (LTRs) of IAPs. In line with the observed variability amongst other IAP-LTRs (Christy *et al.*, 1985), the major differences between the inserts in lines 11/11 and E/1 concerned the putative R regions of the LTRs in which a sequence of 12 nt (CTCTCTTGCTTC) was repeated three times in E/1, but only twice in 11/11. The transcriptional orientation of the LTR insert in line 11/11 was identical to the orientation of the GM-CSF gene, whereas the IAP-LTR in line E/1 was inserted in the opposite orientation.

Evidence for IAP origin of other inserts

The genomes of IAPs display a characteristic restriction pattern (Kuff *et al.*, 1981). A comparison of the postulated internal restriction maps of the inserts in lines 12/10, 12/3 and 6/11 with the map of a full length IAP revealed that the majority of the IAP restriction sites were found in strikingly similar positions in the CSF gene inserts (Figure 8). The inserts, however, were considerably smaller in size than the full-length IAP depicted in Figure 8, suggesting that they represent deleted forms (reviewed by Kuff and Lueders, 1988). The orientation of the putative IAP inserts with respect to the CSF genes was variable. The characterization of the Multi-CSF gene insert in line 5/1 was not sufficiently detailed to allow certain identification as an IAP, although the *Xba*I site is compatible with this possibility (Figure 8).

Although the Multi-CSF gene insert in line 12/3 was likely to be located within the known nucleotide sequence of the gene (Figure 5), PCR amplification of the insert using oligonucleotides with homology to the flanking Multi-CSF gene sequences failed to yield a product of the expected size, probably due to the large size of the insert. However, using a combination of Multi-CSF-specific and IAP-LTR-specific oligonucleotides, the sequences of the 5′ and 3′ junctions between the Multi-CSF gene and the insert could be specifically amplified (Figure 9) adding further support to the conclusion that the insert in line 12/3 was of IAP origin. In contrast to the amplification of the inserts in lines 11/11 and E/1 (Figure 6B), the PCR products at the junction of the insert in 12/3 could only be visualized after hybridization (Figure 9B). Figure 9A shows that the ethidium bromide stained gel was dominated by bands which appeared to be generated by single LTR-specific oligonucleotides (left hand two lanes), suggesting that pairs of IAP elements are arranged in close proximity to each other in a head-to-head or tail-to-tail conformation. Localization in clusters is a well-known feature of IAP genomes (Kuff and Lueders, 1988).

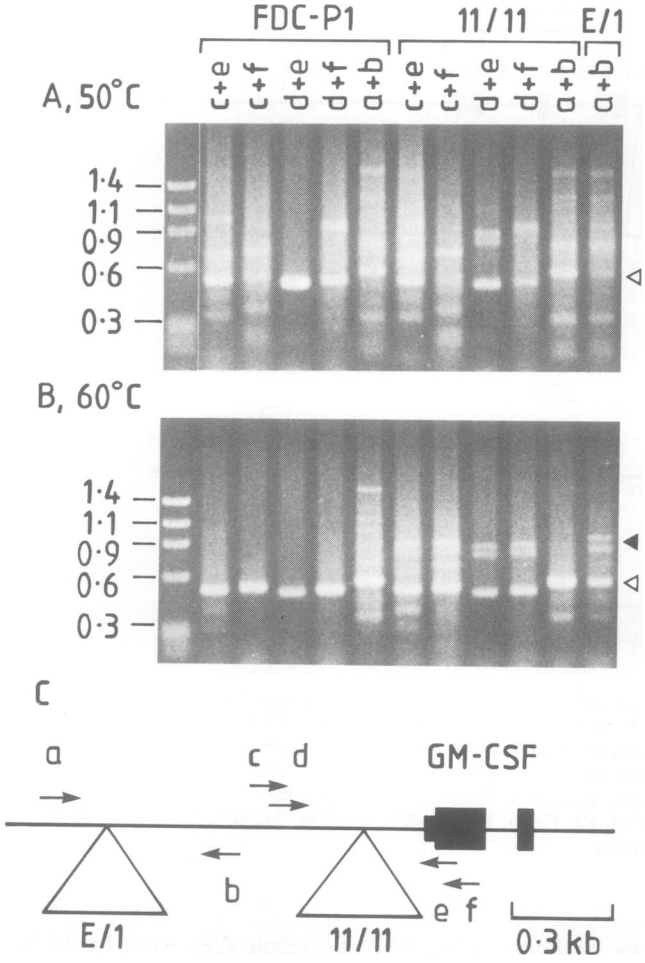

Fig. 6. PCR amplification of the GM-CSF gene inserts in factor-independent lines 11/11 and E/1. Genomic DNA (1 μg) from lines 11/11, E/1 or control FDC-P1 cells was subjected to PCR amplification using GM-CSF gene-specific oligonucleotides spanning the putative insertion sites (**C**). The reactions were performed at 50°C (**A**) or 60°C (**B**) annealing temperatures and the PCR products electrophoresed in agarose gels and stained with ethidium bromide. The type of template DNA and the PCR oligonucleotides used are indicated above each lane. See Materials and methods for sequences of oligonucleotides. Open triangles denote bands corresponding to the PCR products of the unrearranged alleles, and the closed triangle indicates the double-bands corresponding to the PCR products of the alleles containing an insert. ΦX174 DNA digested with *Hae*III was used as size markers.

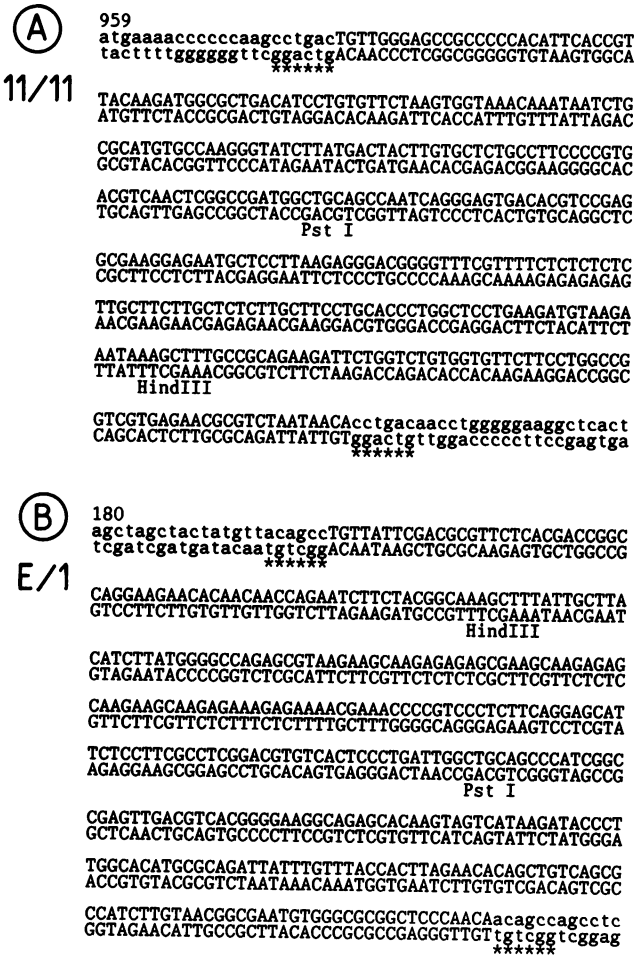

Fig. 7. Nucleotide sequence of the GM-CSF gene inserts in factor-independent lines 11/11 (**A**) and E/1 (**B**). Capital letters designate the sequence of the inserts and lowercase letters the sequence of the flanking GM-CSF gene. Both sequences are given in the transcriptional orientation of the GM-CSF gene, and the numbers at the start of each sequence correspond to the respective nucleotide positions in the GM-CSF sequence published by Miyatake *et al.* (1985a). Note that the entire sequence of the fragment generated with the PCR oligonucleotides (Figure 6) is not shown here. Asterisks indicate direct repeats at the insertion sites. The indicated *Pst*I and *Hin*dIII restriction sites are characteristic of IAP-LTRs (Kuff *et al.*, 1981), and the sequences encompassing these sites were used to design the IAP-specific PCR oligonucleotides used in Figure 9.

Discussion

We have previously described a system for the *in vivo* generation of leukemogenic variants of the continuous hemopoietic cell line FDC-P1. In this system, approximately one third of such variants produce either GM-CSF or Multi-CSF. We describe here the genetic lesions associated with aberrant growth factor gene expression in these latter cell lines. Six out of seven GM-CSF or Multi-CSF-producing FDC-P1 variants analyzed showed a rearrangement in one of the alleles of the corresponding CSF genes. Only one of the CSF-producing lines, which secreted extremely low levels of Multi-CSF, failed to show a rearrangement with the probes and restriction digests used, possibly because the genetic alteration is at some distance from the CSF coding region. In all five cases that were studied in detail, the lesion in the CSF gene appears to be due to insertion of an IAP element in the 5′-flanking region. Since IAP transposition has repeatedly been associated with the activation of genes close to the insertion site (Rechavi *et al.*, 1982; Ymer *et al.*, 1985; Stocking *et al.*, 1988), it appears likely that the constitutive CSF gene activation in the cell lines described here was also causally related to the IAP genomes. It is unclear whether gene activation was due to interference by the inserts with *cis*-acting regulatory elements or provision of enhancer or promoter elements, mechanisms invoked to explain IAP-related gene activation in other cases (reviewed by Kuff and Lueders, 1988).

IAPs are defective retroviruses whose proviral elements are represented ~1000 times in the haploid mouse genome (reviewed by Kuff and Lueders, 1988). Transposition of IAPs has only been sporadically observed in mouse tumor cells. While some of these mutations appeared to be irrelevant to the malignant phenotype of the cells (Hawley *et al.*, 1984; Greenberg *et al.*, 1985), others were possibly

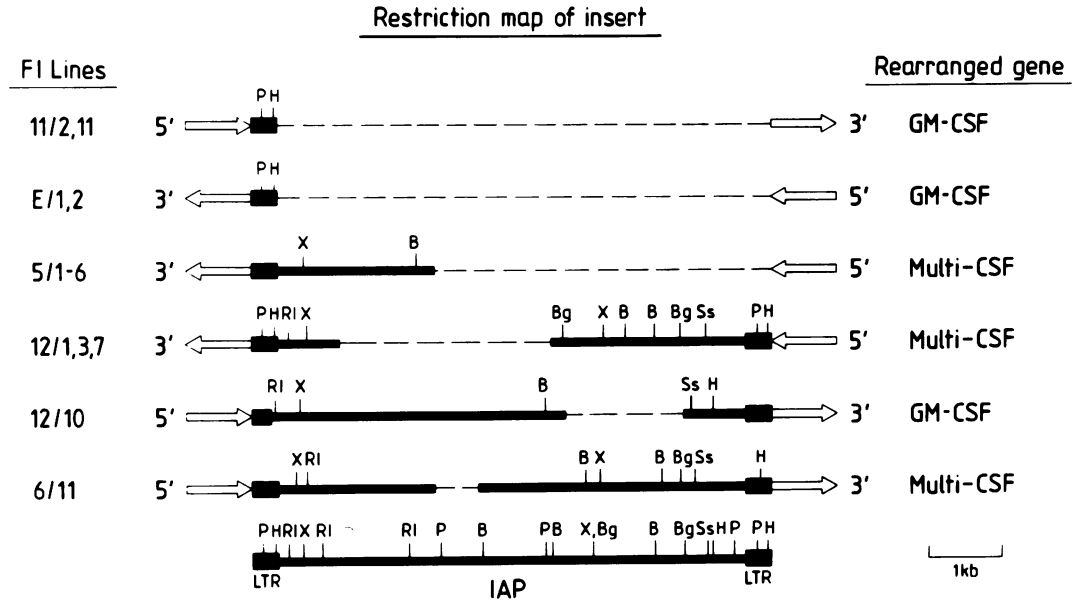

Fig. 8. Comparison between the restriction map of a full-length IAP genome and the restriction maps of CSF gene inserts in factor-independent FDC-P1 variants. The IAP restriction map based on data of Kuff *et al.*, (1981) and Mietz *et al.*, (1987) is shown at the bottom. Dashed lines designate postulated deletions, and open arrows the transcriptional orientation of the respective CSF genes. The restriction maps of the inserts are incomplete, since they were established using hybridization probes with specificity for the adjacent CSF genes. Several of the indicated restriction sites in the full-length IAP are variable among different IAP elements characterized (Kuff *et al.*, 1981; reviewed by Kuff and Lueders, 1988). For abbreviations of restriction endonucleases see legend to Figure 5.

of pivotal importance in the transformation process, like the activation of c-*mos* in plasmactyomas (Rechavi *et al.*, 1982; Canaani *et al.*, 1983; Cohen *et al.*, 1983; Gattoni-Celli *et al.*, 1983), the activation of the Multi-CSF gene in the WEHI 3B myeloid cell line (Ymer *et al.*, 1985, 1986), or the activation of the GM-CSF gene in a derivative of the D35 cell line, a factor-dependent cell line with similar properties to FDC-P1 (Stocking *et al.*, 1988).

IAPs can be sub-divided into different types depending on the size of the genome (Kuff and Lueders, 1988) or the length and structure of the LTRs (Christy *et al.*, 1985). While the overall size of IAP genetic elements involved in somatic mutations ranges from single isolated LTRs (lines 11/11 and E/1 in our study) to full-length 7.2 kb IAPs (Hawley *et al.*, 1984), it is intriguing that all of these transposed IAPs have short LTRs (335–366 nt) with a highly conserved sequence of 23 nt at the 5′ end of the putative R region, which is generally the most variable part of the LTR (Christy *et al.*, 1985). The sequence GTTT-TCTCTCTCTCTTGCTTCTT is almost identical in all 11 LTRs which have so far been reported to be involved in somatic mutations, with only minor variations occurring at positions 5, 9, 10 and 23 (Canaani *et al.*, 1983; Hawley *et al.*, 1984; Stocking *et al.*, 1988; E/1 in this study). Although well represented in the mouse genome (Christy *et al.*, 1985; Man *et al.*, 1987) and apparently involved in germline mutations (Burt *et al.*, 1984), IAPs with long LTRs (~480 nt) or IAPs whose nucleotide sequence in the beginning of the R region differs from that mentioned above, have not yet been observed in somatic rearrangements. It is therefore tempting to speculate that the different types of IAPs vary in their capacity to transpose or activate proximal genes, and that this capacity may in part be determined by the structure of the LTR. Preferential transposition of certain IAP subsets was also observed by Shen-Ong and Cole (1984) when they studied the *de novo* integration of IAP genomes in mouse plasmacytomas.

The origin of the inserted IAP genomes and the mechanisms of integration remain speculative. Due to a defective *env* gene, IAPs cannot be transmitted horizontally from cell to cell (reviewed by Kuff and Lueders, 1988), and it is likely therefore that the transposed IAP elements originated in the FDC-P1 cells themselves, rather than the irradiated host mice. Irradiation, however, is known to activate endogenous viruses (reviewed by Pincus, 1980), and one cannot exclude the possibility that the IAP genome was ferried as a pseudotype in the virion coat of a second retrovirus from host animal to the FDC-P1 cells (reviewed by Weiss, 1982). Since to date, IAP genome transpositions have predominantly been observed in tumor cells that contain large numbers of A-particles (reviewed by Kuff and Lueders, 1988), it would be of interest to ascertain whether FDC-P1 cells express IAP genes, and whether such expression is modified by the irradiated host environment. Although type C particles were described in an analysis of an *in vitro* FDC-P1 transformation system (Boswell *et al.*, 1987), to our knowledge no studies have yet been performed that specifically address the issue of A-particle gene expression in FDC-P1 cells.

The structure of the IAP genome suggests that integration of IAPs follows the same principles as retroviral insertion (reviewed by Kuff and Lueders, 1988), i.e. via reverse transcription of proviral DNA from an RNA intermediate (reviewed by Temin, 1981; Varmus, 1982). The target site duplications flanking the inserts in lines 11/11 and E/1 are also consistent with this concept. In both cases, however, the size of the inserted IAP element was reduced to a single LTR, suggesting that a large part of the IAP genome had been deleted. Solitary LTRs, both of IAP and retroviral origin, are thought to arise from homologous recombination

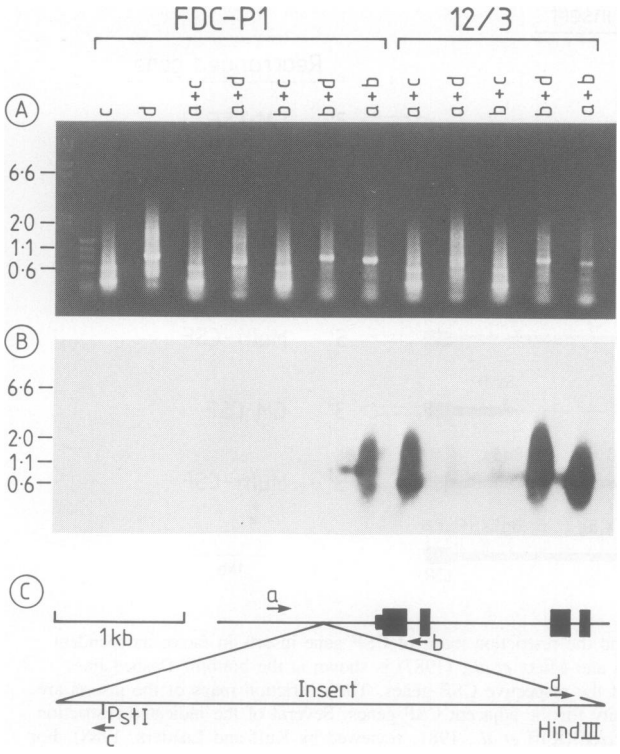

Fig. 9. PCR amplification of the junctions of the insert in the Multi-CSF gene in factor-independent line 12/3. Genomic DNA (1 μg) of line 12/3 or control FDC-P1 cells was subjected to PCR amplification using the oligonucleotides indicated in the diagram (C). **Panel A** shows the ethidium bromide stained gel of the electrophoresed PCR products (reactions performed at 50°C annealing temperature), and **panel B** a Southern transfer hybridized with a radiolabeled DNA probe specific for the 5′-flanking region of the Multi-CSF gene (cf. Figure 5). The type of template DNA and the PCR oligonucleotides used are indicated above each lane. The IAP-specific oligonucleotides were designed on the basis of the IAP inserts in lines 11/11 and E/1 (see Figure 7). See Materials and methods for the sequences of the oligonucleotides. Lambda DNA digested with *Hin*dIII and ΦX174 DNA digested with *Hae*III were used as size markers. Note that both template DNAs allow amplification of the unrearranged allele (oligonucleotide a + b), while the junctions between the Multi-CSF gene and the insert are only demonstrable using 12/3 DNA. See text for interpretation of the stained gel.

between the original 5′ and 3′ LTRs (Payne *et al.*, 1982; Copeland *et al.*, 1983; Wirth *et al.*, 1983; Corcoran *et al.*, 1984; Man *et al.*, 1987) and certainly this appears to be the mechanism of excision of the ecotropic provirus in dilute coat-color revertants (Copeland *et al.*, 1983). If this mechanism also pertains to the IAP-LTRs described here, then lines 11/11 and E/1 must have suffered at least two genetic lesions en route from an FDC-P1 cell, to the cell types that were eventually isolated from the leukemic animals: the primary integration and a subsequent recombination. Moreover, because in our study two out of three IAP insertions in the GM-CSF gene were reduced to isolated LTRs and all sister cell lines from the same mouse showed the same short insert, it appears that reduction of the size of the IAP genome to a solitary LTR resulted in a tumor cell type with a proliferative advantage.

Detection of CSF-specific RNA by PCR technology revealed that various cell lines containing IAP inserts 5′ of the Multi-CSF gene also expressed low levels of GM-CSF RNA. Since the GM-CSF and Multi-CSF genes are located only 10–15 kb apart on the same chromosome, with the Multi-CSF gene lying 5′ of GM-CSF (Lee and Young, 1989; Yang *et al.*, 1988), co-expression of GM-CSF could be due to either co-enhancement, with GM-CSF transcription ensuing from its normal promoter, or a low level of read-through transcription from the Multi-CSF locus. PCR reactions (not shown) using oligonucleotides located 5′ of the GM-CSF promoter (Stanley *et al.*, 1985; Miyatake *et al.*, 1985a) favor the latter possibility.

What role does IAP transposition play in general in the pathogenesis of FDC-P1-derived leukemias? So far our investigations have only focussed on two genes, and in the examples studied gene activation was almost invariably associated with IAP element insertions. Expression of CSF genes, however, can only be demonstrated in about one third of the factor-independent FDC-P1 variants (Dührsen, 1988), leaving the mechanism of transformation in the majority of the leukemias unexplained. It is likely that in these cases other genes involved in the control of cell proliferation are deregulated, and one may speculate that IAP transposition may also be involved in the activation of these genes. Thus, in addition to its potential as a model for studying the generation and development of radiation-induced leukemias, the *in vivo* transformation system described might also provide a system for uncovering novel growth control genes and appears to represent a valuable experimental approach for studying IAP transpositions and their effects on gene regulation.

Materials and methods

Generation of factor-independent FDC-P1 cell lines

The induction of FDC-P1-derived leukemias in DBA/2 mice and the generation of continuous leukemic cell lines have been described in detail elsewhere (Dührsen, 1988); Dührsen and Metcalf, 1988). In brief, FDC-P1 cells were injected i.v. into 0–3.5 Gy irradiated mice. When moribund, the mice were killed, agar cultures prepared from cells of enlarged lymph nodes, and continuous cell lines initiated from individual colonies developing in unstimulated or GM-CSF-stimulated cultures. The FDC-P1 origin of these lines was verified by karyotypic analysis (8 metacentric chromosomes).

Conditioned media from factor-independent lines were collected both from 48-h-old cultures initiated with 5×10^5 cells/ml (standard procedure for quantitation of CSF production) and from overgrown 2–3 week old cultures (in some cell lines CSF production was only demonstrable under these conditions; Dührsen, 1988). To detect very low levels of CSFs, the conditioned media were concentrated 25-fold using Centricon-10 microconcentrators (Amicon, Danvers, MA). Cell lysates were prepared by lysing a pellet of 5×10^6 cells from exponentially growing cultures in 50 μl of 1 M acetic acid. The supernatant was neutralized with 0.25 M NaOH and diluted to a final volume of 1 ml with saline before analysis. The intracellular CSF concentrations were calculated on the basis of an average cell volume of 900 fl which was determined by measuring volumes of nine FDC-P1 variants using a ZM counter and C1000 channelizer (Coulter Electronics Ltd, Luton, Beds., UK) connected to a microcomputer.

CSF bioassays (microwell cultures)

Serial 2-fold dilutions of 5 μl of the conditioned media or cell lysates were added to 10 μl of culture medium containing 200 FDC-P1 cells (which respond to either GM-CSF or Multi-CSF) or 200 32D cl 3 cells (which respond only to Multi-CSF) (Hapel *et al.*, 1984; Metcalf, 1985). The cultures were scored after 48 h of incubation using an inverted microscope and the lowest concentrations stimulating a detectable response (>15 viable cells) determined. These concentrations could be converted to U/ml by the parallel titrations of standard CSF preparations of known activity (50 U/ml is the concentration stimulating half-maximal colony formation in conventional agar cultures of mouse bone marrow cells) (Metcalf, 1984). In microwell cultures, the lowest levels of detectability were 1.5 U/ml for GM-CSF and 1.0 U/ml for Multi-CSF. Mixing experiments using standard CSF preparations and conditioned media from FDC-P1 variants failed to provide evidence for inhibitory material in the media (Dührsen, 1988).

In order to identify the active component in positive conditioned media, the stimulatory activity was neutralized by the addition of serial 10-fold dilutions of a monoclonal rat antibody against murine GM-CSF or a polyclonal rat antiserum against murine Multi-CSF, respectively (both kindly provided by Dr F.Lee, DNAX, Palo Alto, CA). To ensure optimal neutralization conditions, the conditioned media were diluted to concentrations resulting in 50–100% maximal stimulation of FDC-P1 cells in microwell cultures.

RNA detection using the polymerase chain reaction

Total cytoplasmic RNA prepared from 1.5×10^6 cells of each line (Gough, 1988) was included in a 20 μl cDNA synthesis reaction containing: 50 mM Tris–Cl (pH 8.3 at 42°C), 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM of each dNTP, 20 μg/ml oligo-dT(15) and 20 units AMV reverse transcriptase (Boehringer Mannheim). After incubation at 42°C for 40 min, the reaction was terminated by addition of 80 μl distilled water. For PCR amplification, 5 μl cDNA was included in 50 μl reactions containing buffer, dNTPs, 1.25 units Taq polymerase (all supplied by Perkin Elmer Cetus) and 1 μM of each oligonucleotide primer. Three pairs of primers were included simultaneously. GM-CSF: CCTGAGGAG-GATGTGGCTGC and CTGTCCAAGCTGAGTCAGCG, defining a 601 bp cDNA fragment; Multi-CSF: AATCAGTGGCCGGGATACCC and CGAAAGTCATCCAGATCTCG, defining a 300 bp cDNA fragment; and β_2-microglobulin: TTCTCTCACTGACCGGCCTG and CAGTAGACGGTCTTGGGCTC, defining a 308 bp cDNA fragment. The PCR reaction conditions were: 94°C, 90 s; 37°C, 90 s; 72°C, 120 s, for 25 cycles in a Perkin Elmer Cetus DNA thermal cycler. The PCR products were electrophoresed on three parallel 1% agarose gels, transferred to nitrocellulose and hybridized as described below, with nick-translated GM-CSF, Multi-CSF or β_2-microglobulin cDNA probes.

Southern blots

High molecular weight genomic DNA was prepared essentially as described (Gough and Murray, 1982). Aliquots (10–15 μg) of DNA were digested to completion with various restriction endonucleases, electrophoresed in 0.8% agarose gels and transferred to nitrocellulose. Prior to hybridization, filters containing DNA were soaked in 2 × SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.2% Ficoll, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin, 2 mM sodium pyrophosphate, 1 mM ATP, 50 μg/ml denatured salmon sperm DNA and 50 μg/ml *Escherichia coli* tRNA. Hybridization was in the same buffer plus 0.1% SDS at 67°C for ~16 h. The hybridization probes used were: GM-CSF, a 450 bp *Bam*HI–*Pst*I fragment spanning the GM-CSF coding region from cDNA clone pGM3′Δ1.11 (Gough *et al.*, 1987); Multi-CSF, a 1.9 kb genomic *Hin*dIII fragment lying immediately 5′ of the Multi-CSF coding region (see Figure 5). Both were ^{32}P-labeled by nick-translation to a specific activity of $\sim 2-5 \times 10^8$ c.p.m./μg and were included in hybridizations at $\sim 2 \times 10^7$ c.p.m./ml. After hybridization, filters were washed extensively in 2 × SSC, 0.1% SDS at 67°C and finally in 0.2 × SSC at 67°C prior to autoradiography.

PCR amplification of genomic DNA

One microgram aliquots of high molecular weight genomic DNA were included in 50 μl PCR reactions containing buffer, dNTPs and 1.25 units Taq polymerase (Perkin Elmer Cetus) and 1 μM of each oligonucleotide primer (see below). The PCR reaction conditions were: 94°C, 2 min; 40°C, 50°C or 60°C, 2 min; 72°C, 5 min; for 35 cycles in a Perkin Elmer Cetus DNA thermal cycler. The PCR products were fractionated on 1% agarose gels. The GM-CSF gene-specific oligonucleotides used were (letters a–f refer to Figure 6C):

(a) AGCTGAATTCAGTTCCTGATTCCACAG;
(b) AGCTGGATCCTTGGGAGCAGTGAGA;
(c) AGCTGAATTCTGGAATGAGCCACCAG;
(d) AGCTGAATTCAGAGTAGGTAGAGCTT;
(e) AGCTGGATCCCAGGAAAAGTAAATT; and
(f) AGCTGGATCCTGTAGACCACAATGCC.

The Multi-CSF gene-specific oligonucleotides used were (letters a and b refer to Figure 9C):

(a) AGCTGAATTCCCACCTTTGTCCTAG; and
(b) AGCTGAATTCGCCACTGATTGAAGCTT.

The IAP-LTR-specific oligonucleotides used were (letters c and d refer to Figure 9C):

(c) AGCTGGATCCTGCAGCCAATCAGGGA, and
(d) AGCTGGATCCTGCGGCAAAGCTTTATT.

Note that each primer has 17 nt complementary to the respective CSF or IAP genomic sequence and includes a *Bam*HI or *Eco*RI restriction site.

Subcloning and nucleotide sequence analysis

PCR-derived fragments corresponding to the wild-type or the rearranged CSF allele were recovered from agarose gels, digested with *Eco*RI plus *Bam*HI and ligated into the M13mp8 and 9 phage vectors (Messing and Vieira, 1982). Nucleotide sequence analysis by the dideoxy chain termination method was performed using Sequenase reagents (US Biochemicals) universal M13 primers and primers internal to each sequence.

Acknowledgements

We are indebted to Professor D.Metcalf for his critical advice and continuous support throughout these experiments. We thank L.DiRago for excellent technical assistance, Dr N.A.Nicola for provision of purified CSF standards and help with cell lysates, Dr A.Harris for help with measurements of cell volumes and Dr R.Simpson for synthesis of oligonucleotides. This work was supported by grants from The Carden Fellowship Fund of the Anti-Cancer Council of Victoria, the National Health and Medical Research Council, Canberra, and the National Institutes of Health, Bethesda (grants CA-22556 and 25972). U.Dührsen and J.Stahl were supported by scholarships of the Deutsche Forschungsgemeinschaft.

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Received on December 7, 1989; revised on January 26, 1990