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Activation of cMGF expression is a critical step in avian myeloid leukemogenesis

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A non-leukemogenic version of the v-*myb* oncogene causes *in vitro* transformation of avian myeloblasts, which are dependent on chicken myelomonocytic growth factor (cMGF). We have shown that this version of v-*myb*, when combined with the erythroleukemia-inducing v-*erbB* oncogene, is capable of causing a mixed myeloid and erythroid leukemia. Myeloid leukemic cells transformed by this construct produce cMGF. To test whether autocrine growth stimulation via cMGF is the essential contribution of the tyrosine kinase oncogene v-*erbB* in avian myeloid leukemogenesis we constructed another retrovirus containing both the non-leukemogenic v-*myb* and the cMGF cDNA. This virus induced myeloid leukemia at high efficiency. In a third construct we combined v-*myb* with the human EGF-receptor gene. Myeloid cells transformed by this construct could be stimulated to grow by the addition of cMGF or EGF. Growth stimulation with EGF was blocked by a cMGF antiserum indicating that activation of a normal tyrosine kinase-type receptor induces cMGF expression but does not bypass the cMGF requirement. We conclude that cMGF plays a key role in the growth regulation of normal and transformed avian myeloid cells.

Key words: autocrine growth/cytokine/leukemia/oncogene cooperation/v-*myb*

Introduction

Multiple lines of evidence suggest that several genetic alterations contribute to tumorigenesis. Epidemiological and experimental studies have shown that various events such as the deregulated expression of hematopoietic growth factors and mutations in, as well as ectopic expression of, cytoplasmic and nuclear proto-oncogenes can contribute to the formation of leukemias (for review, see Leutz and Graf, 1990).

Avian hematopoietic cells transformed by acutely leukemogenic retroviruses provide a unique system to study oncogene cooperation (Graf and Beug, 1978; Kahn *et al.*, 1986). The nuclear oncogenes v-*myc* and v-*myb* cause the transformation of chicken myeloid cells, the proliferation of which is dependent on the presence of chicken myelomonocytic growth factor (cMGF; Leutz *et al.*, 1984), a cytokine distantly related to the mammalian hematopoietic growth factors G-CSF and IL-6 (Leutz *et al.*, 1989).

In contrast to v-*myc* and v-*myb*, kinase-type oncogenes do not induce the transformation of myeloid cells in the chicken hematopoietic system. Instead, their transforming potential appears to be restricted to the erythroid lineage (Beug *et al.*, 1979; Gazzolo *et al.*, 1979; Kahn *et al.*, 1984, 1986). However, superinfection of v-*myc* or v-*myb* transformed myeloid cells with retroviruses encoding kinase-type oncogenes abrogates their cMGF requirement by inducing endogenous cMGF production and autocrine growth stimulation (Adkins *et al.*, 1984).

A biological relevance for this cooperation between kinase-type and nuclear oncogenes is suggested by the existence of the natural leukemogenic virus isolate MH2. This virus, which has been selected for its high oncogenic potential, contains the v-*myc* oncogene and the serine/threonine kinase encoding oncogene v-*mil* (the chicken homolog of the murine *raf* gene). In contrast to retroviruses which encode either v-*myc* or v-*mil* only, MH2 efficiently induces an acute monocytic leukemia where the leukemic cells produce their own cMGF (Graf *et al.*, 1986). This observation indicated that v-*mil* contributes an essential step to leukemogenesis by inducing autocrine growth in v-*myc* transformed myeloid cells.

In the present study, we asked whether kinase-type oncogenes (we used the v-*erbB* gene of avian erythroblastosis virus as a prototype) can cooperate with the v-*myb* gene in leukemogenesis and, if so, whether the induction of cMGF expression corresponds to the biological effect of the v-*erbB* oncogene. To study this question, we used a non-leukemogenic version of the v-*myb* oncogene to construct myb/erbB and myb/cMGF viruses and investigated their leukemogenic potential. We also constructed a virus containing both v-*myb* and the human epidermal growth factor (EGF) receptor gene (HER; Ullrich *et al.*, 1984) to determine whether in such transformed myeloid cells EGF alleviates the cMGF requirement.

Here we report that v-*erbB* cooperates with v-*myb* during myeloid leukemia development and that it can be functionally replaced by the cMGF gene. In addition, we found that the human EGF-receptor coexpressed with v-*myb* in transformed myeloid cells induces autocrine growth via activation of endogenous cMGF expression in a strictly EGF-dependent manner. These results are compatible with the notion that cMGF activation is the critical effect of tyrosine kinase-type oncogenes contributing to the leukemic potential of v-*myb* transformed avian myeloid cells.

Results

A non-leukemogenic v-myb oncogene in conjunction with v-erbB causes mixed myeloid and erythroid leukemia

To investigate whether a tyrosine kinase-type oncogene can cooperate with v-*myb* in the induction of myeloid leukemias we constructed a retrovirus containing both the v-*erbB* oncogene (Vennström and Bishop, 1982) and v-*myb*^{EEA}

(referred to as v-*myb* in the following), a non-leukemogenic version of the v-*myb* oncogene. A virus encoding this version of v-*myb* alone was already available (Frykberg *et al.*, 1988; Materials and methods). The genomic maps of these viruses, which were called CRmyb-erbB (CR: chicken retrovirus) and CRmyb, respectively, and the proteins they encode are shown in Figure 1. Both constructs expressed a v-*myb* protein of the expected size of 77 kd. In addition, CRmyb-erbB expressed a 69 kd v-*erbB* protein from the spliced subgenomic mRNA (Figure 1B). These viruses also exhibited the expected transformation behavior in bone marrow cultures, CRmyb giving rise to transformed colonies of the myeloid lineage only and CRmyb-erbB transforming both erythroblasts and myeloblasts. Of more than five individually transformed myeloid cell clones tested for each construct, all CRmyb transformed clones were cMGF dependent while those transformed by CRmyb-erbB were not. Factor independence of the latter cells was likely to be due to the induction of cMGF production and autocrine growth (Adkins *et al.*, 1984; unpublished results). In addition, we were able to detect cMGF expression in five out of five clones tested using a PCR technique (unpublished observations).

To determine their leukemogenic potential, two groups of 7-day-old chicks were infected intravenously with the two viruses. As shown in Table I, CRmyb-erbB caused leukemias with an average latency period of 2.5 weeks post-infection. In contrast, CRmyb injected animals did not develop leukemias even after three months. The presence of functional virus in the non-leukemic animals could be demonstrated by the outgrowth from their bone marrow of transformed, cMGF dependent myeloblasts *in vitro* (cf. Figure 1B and see below).

To determine the type of leukemia induced by CRmyb-erbB, cells from the peripheral blood of several leukemic chickens were enriched in a low density fraction ('buffy coat') and examined by cytological and immunofluorescence techniques. To identify transformed erythroid cells we used antibodies to histone H5 and, in some experiments, antibodies to erythroblast cell surface antigen (Ebl Ag); avian myeloid cells were identified by the expression of the 51/2 cell surface antigen detected by the corresponding monoclonal antibody. As summarized in Table II, there were large variations in the percentage of H5 positive cells when buffy coats from different leukemic animals were compared. One animal contained as few as 19% H5 positive cells, others up to 76%. The low proportion of erythroid cells in the buffy coat of animal number 10 suggested that its leukemia consisted predominantly of non-erythroid cells. Indeed, after culturing these cells for one week, to allow death of non-transformed cells, essentially all were found to be myeloid as judged by the lack of expression of erythroid and by the presence of myeloid cell surface antigens (data not shown). In contrast, similar experiments performed with cells of the other four animals tested revealed a mixture of both erythroid and myeloid leukemic cells in various proportions. The erythroid component of the leukemia was caused through the direct action of v-*erbB* (Frykberg *et al.*, 1983). To determine the clonality of leukemic cells, Southern blot hybridizations were performed with cell DNAs from a few animals. Since retroviruses essentially exhibit random integration sites, each transformed clone is 'marked' with a specific proviral integration site. As shown in Figure 2,

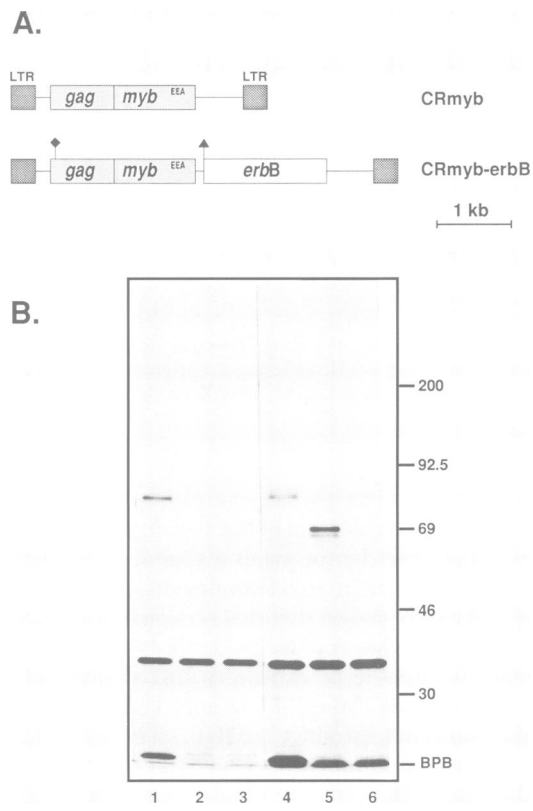

Fig. 1. Genomic structures and proteins of CRmyb and CRmyb-erbB. **A.** Genomic structures. Splice donor and acceptor sites are indicated by a filled square and a triangle, respectively. Details of the virus constructs are given in Materials and methods. **B.** Proteins expressed by myeloid cells transformed by CRmyb (lanes 1, 2 and 3) or by CRmyb-erbB (lanes 4, 5 and 6). Cultured cells from bone marrow of CRmyb animal number 6 and from the buffy coat of CRmyb-erbB animal number 10 were labeled with [^{35}S]methionine, extracts incubated with anti-*myb* (lanes 1 and 4), anti-*erbB* (lanes 2 and 5) or preimmune serum (lanes 3 and 6) and precipitates separated by gel electrophoresis. BPB, bromophenol blue, indicating the front of the gel. Numbers on the right correspond to mol. wt markers (in kd) run in parallel.

Table I. Leukemogenic capacity of a CRmyb erbB and CRmyb viruses

Viral construct	Leukemia incidence	Average latency period (range) in days
CRmyb-erbB	15/15	16.2 (13–20)
CRmyb	0/15	N.R.

N.R., not relevant

all four animals tested exhibited a smear of bands indicative of polyclonality. In addition, they exhibited one to two predominant integration sites, suggesting the occurrence of individual clones which are largely over-represented and which may explain the predominance of leukemic cells from either the erythroid or myeloid lineage.

To determine whether the CRmyb-erbB transformed myeloid cells obtained from leukemic animals are growth factor independent, it was necessary to obtain homogeneous cultures of myeloid cells. For this purpose we seeded leukemic cells from animal number 10 and, as a control, bone marrow cells from CRmyb animal number 6 (prepared two months post-infection) into Methocel containing cMGF.

Table II. Phenotype of leukemic cells obtained after injection of Cmyb erbB virus

Leukemic chick no.	Morphology of leukemic cells[a]			Percent marker-positive cells		
	Ebl	Mbl	Mye	Histone H5[b]	EblAg[c]	51/2Ag[c]
8	+	+	+	32	28	71
10	−	+	+	19	1	99
11	+	−	−	77	60	35
13	+	+	+	60	64	35
19	+	+	+	76	60	40

[a]Phenotype determined after microscopic inspection of Giemsa-stained blood smears: Ebl, erythroblast; Mbl, myeloblast; Mye, promyelocyte
[b]Nuclear immunofluorescence of fixed buffy coat cells
[c]Cell surface immunofluorescence of cells cultured for 1 week after preparation of buffy coat.

Ten days later five transformed colonies derived from each animal were isolated, verified to be myeloid by testing for their 51/2 expression, pooled and their growth kinetics studied with and without cMGF addition. As shown in Figure 3A, myeloid cells transformed by either of the two viruses grew at similar rates in liquid medium supplemented with cMGF. In contrast, CRmyb but not CRmyb-erbB transformed cells stopped growing when cMGF was omitted from the medium (Figure 3B). Similar results were obtained with each of the five transformed clones tested individually (data not shown). To determine whether CRmyb-erbB transformed myeloid cells produce cMGF, conditioned medium was prepared from the pool of myeloid leukemic cells of animal number 10 and titrated on factor-dependent E26-transformed myeloblasts. Supernatants of CRmyb transformed cells from animal number 6 were also tested. As shown in Figure 3C, the supernatants of CRmyb-erbB but not CRmyb cells contained significant amounts of growth factor activity. This activity could be neutralized with antibodies to cMGF (data not shown).

v-myb transformed myeloid cells expressing the human EGF receptor (HER) can be stimulated to cMGF secretion and autocrine growth by EGF

Our results showed that v-*erbB* confers growth factor independence to v-*myb* transformed chicken myeloid cells by inducing cMGF production and autocrine growth stimulation. We determined next, whether ligand activation of ectopically expressed human EGF receptor abolishes the cMGF requirement of v-*myb* transformed myeloid cells. Accordingly, we generated a virus, termed CRmyb-HER, which directs the synthesis of both v-*myb* and human EGF receptor. This was done by exchanging the v-*erbB* oncogene in the CRmyb-erbB virus with the HER gene (Ullrich *et al.*, 1984; Khazaie *et al.*, 1988; Figure 4A).

When used to infect bone marrow cells in the presence of cMGF, CRmyb-HER efficiently transformed myeloid cells. CRmyb-HER is also capable of transforming erythroid cells, provided that EGF is supplied with the medium (unpublished observations). As shown in Figure 4B, myeloid cells transformed by CRmyb-HER expressed myeloid cell surface markers and were also stained by antibodies to the EGF receptor. The finding that the latter antigen is subjected to down regulation after EGF administration (Figure 4f) supports the notion that the cells express functional EGF receptor.

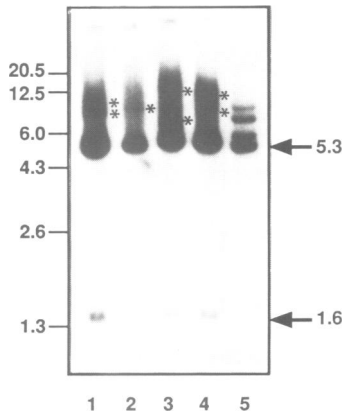

Fig. 2. Proviral integration sites in DNA from leukemic cells of CRmyb-erbB-infected animals. DNA was isolated from leukemic cells of animal 2, 5, 7 and 10 (lanes 1−4) and of a pool of three erythroid clones transformed *in vitro* by CRmyb-erbB (lane 5). A Southern blot analysis was performed with *Hin*dIII-digested DNA hybridized to a *myb* probe. The arrows on the right point to the endogenous c-*myb* bands, those on the left indicate size markers run on the same gel. Values represent kilobases.

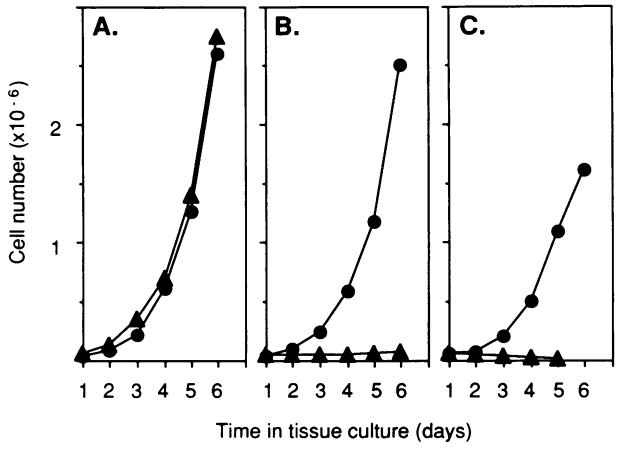

Fig. 3. cMGF dependence of and production by CRmyb and CRmyb-erbB cells. Cells were grown either in the presence (**A**) or absence (**B**) of crude cMGF and cell counts determined at daily intervals (**C**). Factor production as determined by testing the effect of 50% conditioned medium from the above cells (grown in the absence of cMGF for 48 h) on the growth kinetics of cMGF-dependent E26-transformed myeloid cells. △, CRmyb cells; ●, CRmyb-erbB cells.

CRmyb-HER transformed myeloid cells, but not CRmyb transformed cells, were able to grow in the absence of cMGF when they were supplied with 20 ng/ml EGF (data not shown). This observation can be explained by the capacity of the activated EGF receptor either to bypass the effect of cMGF or to induce autocrine growth via the activation of cMGF expression. To distinguish between these possibilities, we determined the effect of cMGF antibodies on the growth stimulation induced by EGF in a thymidine incorporation assay. As control cells we used myeloblasts transformed by a recombinant retrovirus, termed CRmyb-cMGF, which encodes the cMGF cDNA in combination with v-*myb* (see below). As shown in Figure 5, cMGF antibodies inhibited EGF-induced growth in CRmyb-HER transformed cells by >80%. As expected, antibodies to cMGF also inhibited the growth of CRmyb-cMGF transformed cells to

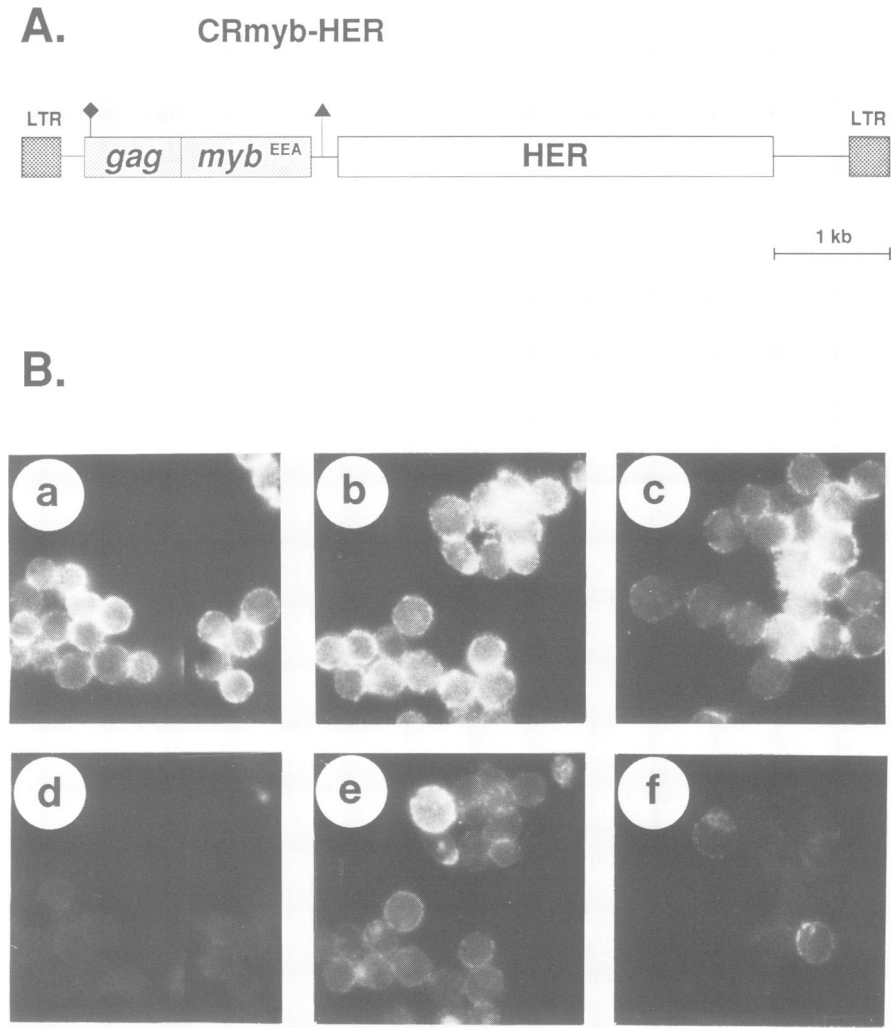

Fig. 4. Genomic structure of CRmyb-HER virus and proteins produced. **A**. See legend to Figure 1A. **B**. Expression of cell surface antigens in CRmyb (a and d) and CRmyb-HER (b, c, e and f) transformed myeloid cells. Cells were stained with rabbit anti-myeloblast serum and TRITC-labeled goat anti-rabbit antibody (a, b and c). The same cells were also stained with an anti-EGF receptor monoclonal antibody and FITC-labeled rabbit anti-mouse antibody (d, e and f). Cells in c and f were pretreated for 16 h with 20 ng/ml of human EGF.

a similar extent. The observed growth inhibition was due to specific neutralization of cMGF and not to a toxic effect of the antiserum since it could be reversed with a high dose of recombinant cMGF. These experiments show that ligand stimulation of the EGF receptor ectopically expressed in myeloid cells does not bypass the cMGF signal. Instead, the EGF receptor induces cMGF production and autocrine growth stimulation as observed before with its truncated avian version, the v-*erbB* oncogene.

A viral construct encoding cMGF together with v-myb efficiently causes myeloid leukemia

Our results show that a retrovirus expressing v-*erbB* in addition to v-*myb* can cause myeloid leukemia in chickens. Moreover, constitutive or ligand induced tyrosine kinase activities render v-*myb* transformed myeloblasts independent of exogenously added cMGF by autocrine growth stimulation. We therefore tested whether expression of the cMGF gene in conjunction with v-*myb* is sufficient to induce leukemia. For this purpose we constructed another recombinant retrovirus, designated CRmyb-cMGF, which in addition to v-*myb* expresses correctly glycosylated cMGF proteins from a subgenomic messenger (Figure 6). As a control, we constructed a virus termed CRneo-cMGF, by replacing the v-*myb* gene with the *neo* gene (Figure 6). Bone marrow cells transformed *in vitro* by CRmyb-cMGF expressed the 51/2 antigen and, as for CRmyb-erbB or CRmyb transformed myeloid cells, often exhibited granules characteristic of promyelocytes (Introna *et al.*, 1990). As expected, these myeloid cells grew in a factor independent fashion and produced similar levels of cMGF as myeloid cells transformed by CRmyb-erbB, i.e. 10–60 units/ml over a period of 24 h (data not shown). As already mentioned above, the growth of these cells could be inhibited by antibodies to cMGF (Figure 5). In contrast, CRneo-cMGF did not transform bone marrow cells but induced growth factor independence when used to superinfect CRmyb transformed myeloblasts (data not shown).

To investigate whether CRmyb-cMGF induces leukemia the virus was injected into a group of 1-week-old chicks. In parallel, a second group of chicks was injected with the CRneo-cMGF construct. The results of this experiment are summarized in Table III and Figure 7. CRmyb-cMGF but not CRneo-cMGF induced a myeloid leukemia with an

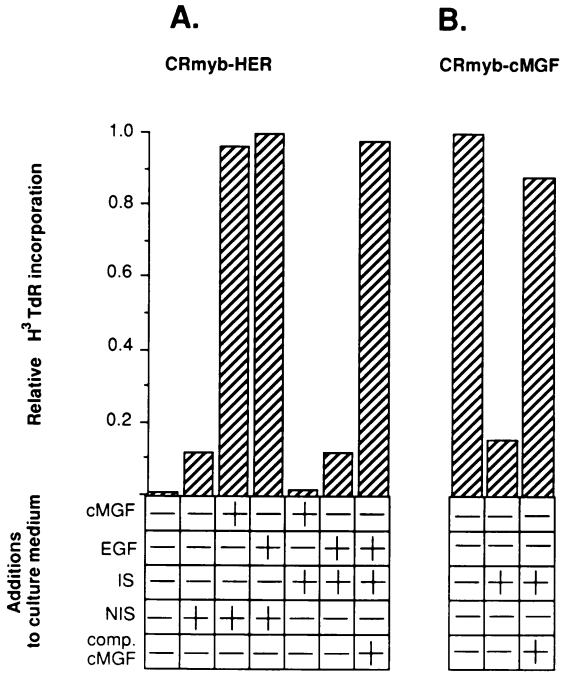

Fig. 5. Demonstration of growth factor independence and autocrine cMGF production in CRmyb-HER and CRmyb-cMGF transformed myeloid cells. Myeloid cells transformed by either CRmyb-HER or CRmyb-cMGF were seeded on microtiter plates containing assay medium with the following additions: 1% preimmune serum (NS), 1% anti-cMGF antiserum (IS), 5 units/ml native cMGF (cMGF), 500 units/ml recombinant cMGF to compete with antiserum (comp. cMGF) or 20 ng/ml EGF (EGF). Additions are indicated by +, omissions by −. After culture for 2 days, cells were pulse-labeled for 2 h with tritiated thymidine as described (Leutz *et al.*, 1984) and the averages of duplicates were plotted after normalization relative to maximal thymidine incorporation (85 000 c.p.m. in fourth sample of panel **A**; 35 000 c.p.m. in first sample of panel **B**).

Table III. Leukemogenic capacity of CRmyb cMGF and CRneo cMGF viruses

Viral construct	Leukemia incidence	Average latency period in days (range)	Phenotype of leukemic cells
CRmyb cMGF	10/11	27.2 (19−36)	Myeloblasts/ promyelocytes
CRneo cMGF	0/19	N.R.	N.R.

N.R., not relevant

average latency of ~4 weeks. The leukemic animals exhibited large numbers of promyelocyte-like cells in their peripheral blood (Figure 7) and in their (enlarged) spleens. In contrast to the results obtained with CRmyb-erbB, no transformed erythroblasts could be detected among the leukemic cells transformed by CRmyb-cMGF. These results demonstrate that the cMGF gene is capable of functionally replacing a tyrosine kinase type oncogene in inducing myeloid leukemia when coexpressed with v-*myb*.

Discussion

The results presented indicate that the capacity of kinase-type oncogenes to cooperate with nuclear oncogenes in avian myeloid leukemogenesis can be explained solely on the basis of their ability to induce cMGF expression followed by autocrine growth. This is most clearly demonstrated by the

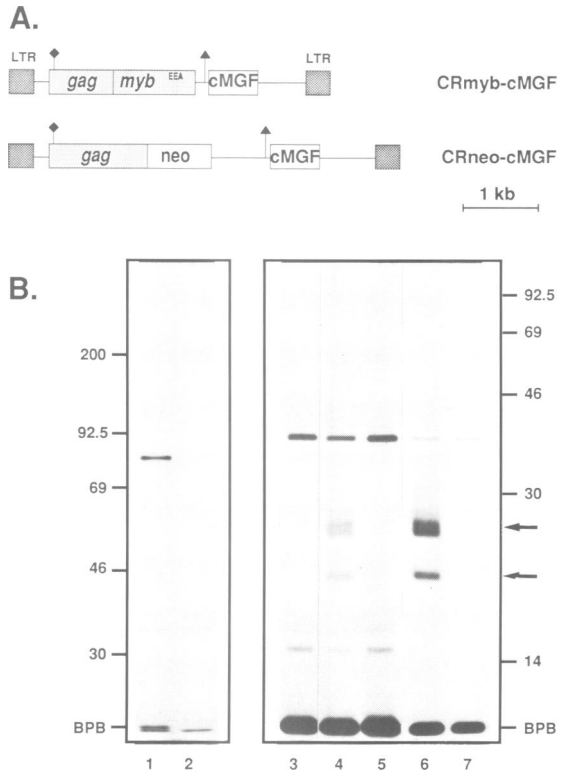

Fig. 6. Genomic structure of and proteins produced by CRmyb-cMGF and CRneo-cMGF. **A**. See legend to Figure 1A. **B**. Protein expressed by myeloid cells transformed by CRmyb-cMGF (lanes 1, 2, 3 and 4) or by CRmyb (lane 5) and, as a positive control, LPS stimulated HD11 cells (lanes 6 and 7). [^{35}S]Methionine labeled cell extracts were incubated with anti-*myb* serum (lane 1), preimmune serum (lanes 2, 3 and 7) or anti-cMGF serum (lanes 4, 5 and 6). Numbers on the left and right indicate positions of mol. wt markers (in kd) run in parallel. Upper arrow corresponds to the N-glycosylated forms of cMGF; lower arrow corresponds to the O-glycosylated forms of cMGF (see also Leutz *et al.*, 1988).

finding that a recombinant virus containing both a non-leukemogenic v-*myb* and the cMGF gene efficiently induces an acute myeloid leukemia. However, our studies do not rule out the possibility that, besides the induction of cMGF expression, kinase-type oncogenes elicit additional, more subtle changes in v-*myb* transformed myeloid cells which might contribute to leukemogenesis. The observation that CRmyb-erbB virus appears to cause leukemia within a shorter latency period than CRmyb-cMGF might argue in favor of such a possibility. However, it is difficult to compare the latencies of these constructs since CRmyb-erbB but not CRmyb-cMGF acts along two lineages.

It is possible that cMGF induction in v-*myb* transformed myeloid cells by overexpression of activated tyrosine kinases is an indirect effect, resulting from the induction of more differentiated cells which produce the factor. In support of this possibility is the observation that at least some v-*myb* transformed myeloid cells superinfected with a v-*erbB* containing virus acquire macrophage-like properties such as adherence, size and increased motility in agar (unpublished observations). In addition, we have demonstrated in earlier work that myeloblasts transformed by a temperature sensitive mutant of E26 virus can be induced to produce cMGF by shifts to the nonpermissive temperature, a treatment which triggers their differentiation into macrophage-like cells

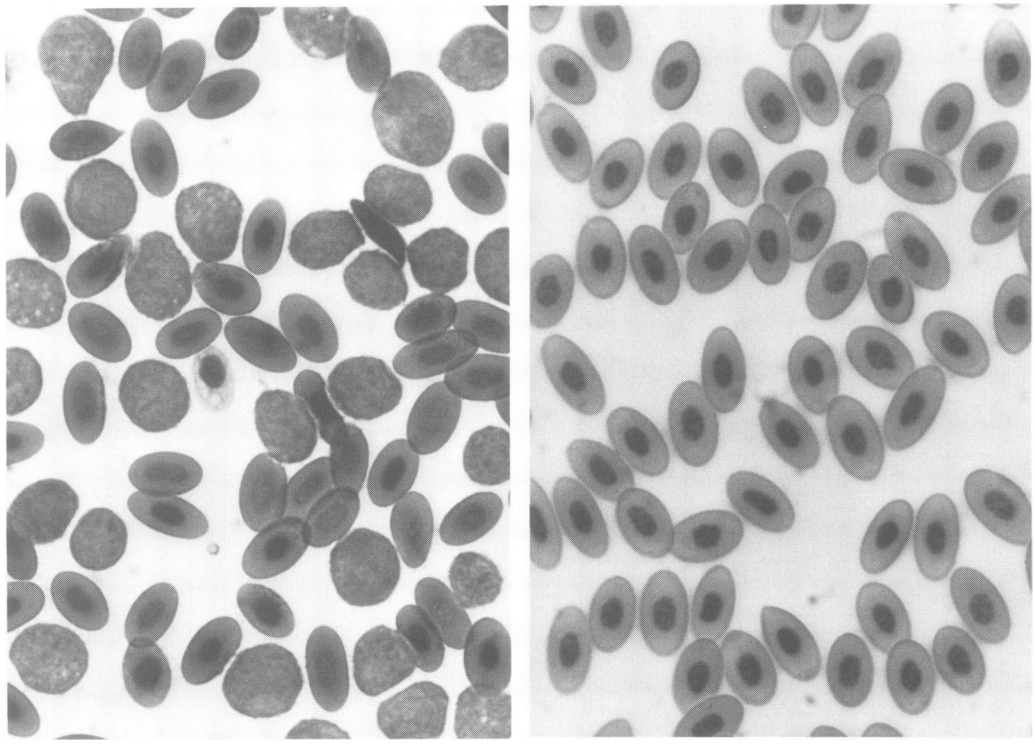

Fig. 7. Blood smears from a leukemic, CRmyb-cMGF injected (left) and from a normal chicken (right).

(Beug *et al.*, 1984). Finally, normal avian macrophages produce low but detectable levels of cMGF (unpublished observations).

The data shown here and in earlier work (Graf *et al.*, 1986) indicate that a minimum of two events is required to cause avian myeloid leukemias efficiently: (i) the expression in myeloid target cells of either the v-*myb* or v-*myc* oncogenes and (ii) the induction of cMGF expression. The finding that the CRmyb-cMGF is leukemogenic, together with the demonstration that growth of cell cultures transformed by this construct can be suppressed by cMGF antibodies, strongly suggests that cMGF expression and autocrine growth stimulation play a causative role in the development and in the maintenance of the leukemic phenotype. Interestingly, the concomitant acquisition by myeloid cell lines of leukemogenicity and growth factor independence via autocrine rather than short-circuit mechanisms seems to apply also to mammalian cells (Stocking *et al.*, 1988; Browder *et al.*, 1989). How then does the avian myeloblastosis virus (AMV), which contains a v-*myb* oncogene only (Klempnauer *et al.*, 1982), cause an acute myeloid leukemia (Moscovici, 1975)? Preliminary evidence suggests that AMV transformed cells exhibit a reduced cMGF requirement, perhaps due to paracrine stimulation by cMGF secreted from macrophage-like cells which spontaneously emerge in AMV-transformed myeloblast cultures (unpublished observations). Such a speculation is supported by the finding that macrophage-like cells derived from E26 transformed myeloblasts produce cMGF (Beug *et al.*, 1984).

Tyrosine phosphorylation has been proposed to play an important role in signal transduction mechanisms triggered by hematopoietic growth factors (Morla *et al.*, 1988; Isfort and Ihle, 1990). However, neither the v-*erbB* oncogene nor any of the other kinase-type oncogenes tested were found to be able to directly bypass the cMGF requirement of transformed avian myeloid cells (Adkins *et al.*, 1984). In addition, we found here that in CRmyb-HER cells EGF does not short-circuit the requirement for cMGF but induces cMGF expression which in turn stimulates cell proliferation. Thus, the cMGF gene can be activated by the tyrosine kinase activity of either a mutated, constitutively activated or a ligand induced EGF receptor. These data also suggest that the cMGF receptor is not a tyrosine kinase itself or, if it is, that it has an unusual substrate specificity. In this context it should be pointed out that most of the known hematopoietic growth factor receptors are not members of the tyrosine kinase family (for review, see Cosman *et al.*, 1990). We are currently attempting to clone the cMGF receptor.

The observation that cMGF is activated by kinase-type oncogenes in myeloid but not in erythroid cells (Beug *et al.*, 1984; unpublished results) suggests the requirement of a tissue-specific component. Therefore, one of the challenges for the future is to determine at the molecular level how kinase-type oncogenes induce cMGF expression and to identify the postulated myeloid cell specific component necessary for its activation.

Materials and methods

Construction of recombinant retroviruses

Recombinant retroviruses were constructed using standard procedures (Maniatis *et al.*, 1982). The proviral genomes are contained in the plasmid vector backbone pCR1 (or derivatives thereof) which confers resistance to the drug G418 (Jansson *et al.*, 1987).

The construction of CRmyb, formerly named ts21wtEEA, has been described earlier (Frykberg *et al.*, 1988). Briefly, the E26 *gag-myb* sequence up to the *Sal*I site was combined with the *Sal*I–*Xba*I DNA fragment

containing the 3'-terminal 448 nt of the AMV *myb* gene and inserted as an *XhoI*−*XbaI* fragment into a derivative of pCR-1 named pCR-X.

CRmyb-erbB was constructed from pCR Pax (Choi, 1986) by replacing the *XhoI*−*XbaI gag-erbA* DNA fragment from the genome of ts167AEV ES4 (Choi *et al.*, 1986) with the corresponding *gag-myb* DNA fragment of CRmyb. The subsequent exchange of ts167*erb*B with wt*erb*B was verified by DNA sequencing.

To obtain CRmyb-HER the *gag-myb* containing *XhoI*−*BsmI* DNA fragment of CRmyb-erbB and the *BsmI*−*Hin*dIII DNA fragment of HER-C (which contains the modified splice acceptor site of AEV-ES4, Khazaie *et al.*, 1988) were subcloned into the HER containing *XhoI*−*Hin*dIII plasmid vector backbone fragment of HER-C (Khazaie *et al.*, 1988). From this construct HER is transcribed as a spliced, subgenomic mRNA containing two open reading frames. The first is a short open reading frame initiating in *gag* and terminating downstream of the splice acceptor site. The termination codon is followed by the authentic HER initiation codon where translation reinitiates.

To generate CRmyb-cMGF, the 690 bp *Eco*RI−*Ssp*I DNA fragment of the cMGF cDNA (Leutz *et al.*, 1989) was subcloned into the *Eco*RI−*Sma*I sites of the Bluescript plasmid (Stratagene) to generate pFP-1. This removed the ATTTA repeats contained in the 3' untranslated cMGF sequence. The *Hin*dIII−*Xba*I DNA fragment from pFP-1 encoding cMGF was then used to replace the *Hin*dIII−*Xba*I DNA fragment containing the HER gene of CRmyb-HER.

CRneo-cMGF was constructed by replacement of the *XhoI*−*Sal*I gag-myb DNA fragment of CRmyb-cMGF by the *XhoI*−*Sal*I gag-neomycin phosphotransferase DNA fragment (neo). This exchange leaves the 448 3'-terminal nucleotides of *myb*, which are, however, not translated.

Transfection of chicken embryo fibroblasts

Primary chicken embryo fibroblasts (CEFs) from 11-day old embryos were transfected with 10 μg of recombinant provirus DNA in the context of a G418-resistance conferring plasmid vector backbone and 10 μg of RAV-1 DNA as described earlier (Frykberg *et al.*, 1988). Forty-eight hours later G418 (Gibco) was added (800 μg/ml) and the cells were cultured until the G418 resistant colonies had grown to confluency.

Cell growth and virus assays

CEFs were propagated in Dulbecco's modified Eagle's medium (Gibco) plus 8% fetal calf serum, 2% chicken serum and 10 mM HEPES, pH 7.4. Myeloblast cultures were supplemented with 5−20 units/ml of recombinant cMGF (Leutz *et al.*, 1989) or crude cMGF from concanavalin A stimulated spleen cells (Beug *et al.*, 1982a; Leutz *et al.*, 1984) as indicated.

In order to obtain transformed myeloblasts, 20×10^6 chicken bone marrow cells (prepared from 3- to 10-day-old chicks according to Graf, 1973) were cocultivated for two days with 5×10^5 G418 resistant, virus producing CEFs in growth medium with crude cMGF (myeloid conditions, Radke *et al.*, 1982). Subsequently, non-adherent cells were removed and reseeded in growth medium with crude cMGF. Rapidly proliferating transformed myeloid cells appeared after 8−10 days of culture. The supernatants of these cultures were collected, Millipore filtered and frozen at −70°C as a source of virus (in the case of the non-transforming CRneo-cMGF virus supernatants from G418 resistant CEFs were collected).

To determine virus titers bone marrow cells were infected at different dilutions and seeded in methyl cellulose (Methocel) containing medium with cMGF. The number of transformed colonies was determined after 7−10 days as described (Graf *et al.*, 1981). Since the CRneo-cMGF virus does not exhibit any transforming potential, virus suspensions were either titrated for their ability to induce G418 resistance in fibroblasts or to abrogate the cMGF requirement of E26 transformed myeloblasts.

Isolation and analysis of genomic DNA

Peripheral blood of diseased animals was collected and genomic DNA extracted according to standard procedures (Maniatis *et al.*, 1982). Equal amounts of DNA were digested with *Hin*dIII to completion, run on 0.8% agarose gel, transferred to a nylon membrane and UV crosslinked. Filters were prehybridized for 4 h at 63°C in 0.5 M Na-phosphate, pH 7.2, 7% SDS (Church and Gilbert, 1984). For hybridization this mixture was replaced by a fresh solution containing a randomly primed v-*myb* *XhoI*−*Xba*I DNA fragment (5×10^8 c.p.m./μg) and incubated for 18 h at 63°C. The filter was washed twice at room temperature in 2 × SSC, 0.1% SDS to remove excess labeled probe and 2 × 30 min in 0.2 × SSC, 0.1% SDS at 65°C and exposed to X-ray film.

Assays for cMGF dependence and cMGF production

Cell growth kinetics were determined by seeding in duplicate 0.5×10^6 cells/ml into 35 mm dishes in 2 ml of growth medium with or without cMGF. Cells were counted at daily intervals using a Coulter counter. The cells were diluted to 0.5×10^6 cells/ml in fresh medium when the cell number exceeded 2×10^6 cells/ml. All other cells received a partial medium change every second day. The cumulative cell number was obtained by multiplication of the actual cell number with the corresponding dilution factor.

For the cMGF bio-assay, cells were cultured for two days and then pulse-labeled with tritiated thymidine, as described earlier (Leutz *et al.*, 1984). For the neutralization experiments, anti-cMGF antiserum or pre-immune serum were added at a 1:100 dilution at the beginning of the experiment and one day later.

Conditioned media to be tested were heat-inactivated for 30 min at 56°C to destroy infectious virus, cleared by centrifugation at 20 000 *g* for 30 min and filter sterilized (0.45 μm). Subsequently, cell growth kinetics of indicator cells (E26 transformed myeloblasts) were determined in response to the respective media (see above).

Immunoprecipitation

Immunoprecipiation of [^{35}S]methionine labeled cell extracts or supernatants was performed as described earlier (Radke *et al.*, 1982; Leutz *et al.*, 1988). Antisera against Myb (Ness *et al.*, 1987), ErbB (Hayman *et al.*, 1983) and cMGF (Leutz *et al.*, 1984) have been described.

Immunofluorescence

To determine the lineage of transformed cells, they were reacted with rabbit anti-erythroblast antiserum and rabbit anti-myeloblast antiserum (Beug *et al.*, 1979) or the mouse monoclonal antibody 51/2 (Kornfeld *et al.*, 1983) in an indirect double immunofluorescence assay as described earlier (Beug *et al.*, 1982b). Histone H5, a marker of erythroid cells, was detected with corresponding rabbit antibody on fixed cells as described earlier (Beug *et al.*, 1979). For the detection of human EGF-receptor on the surface of transformed myeloblasts the mouse R1 monoclonal antibody (Khazaie *et al.*, 1988) was used.

Cytochemical analysis

Blood smears were prepared from the peripheral blood of infected chickens, methanol fixed, stained with benzidine (McLeod *et al.*, 1974; Beug *et al.*, 1982b) and counterstained with a Giemsa-like stain (Diff Quick, Harleco) as described earlier (Beug *et al.*, 1982b). Tissue culture cells were collected onto coverslips by cyto-centrifugation and processed like cells from blood smears.

Animal experiments

Seven-day-old chicks of the Spafas flock maintained in Heidelberg were used for all the experiments. Chicks were inoculated intravenously with 0.1 ml undiluted virus stocks, containing 10^3-10^4 infection units/ml. Animals were monitored for the onset of leukemia by examination of blood smears twice weekly starting from 1 week post-infection as described (Graf *et al.*, 1986). To obtain leukemic cells for tissue culture experiments, peripheral blood of moribund chicks was collected by heart puncture and buffy coat cells prepared as described (Radke *et al.*, 1982).

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