The evolution of apical dominance in maize

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The domestication of crop plants has often involved an increase in apical dominance (the concentration of resources in the main stem of the plant and a corresponding suppression of axillary branches)[1]. A striking example of this phenomenon is seen in maize (*Zea mays* spp. *mays*), which exhibits a profound increase in apical dominance compared with its probable wild ancestor, teosinte (*Zea mays* ssp. *parviglumis*)[2]. Previous research has identified the *teosinte branched1* (*tb1*) gene as a major contributor to this evolutionary change in maize[3]. We have cloned *tb1* by transposon tagging and show here that it encodes a protein with homology to the *cycloidea* gene of snapdragon[4]. The pattern of *tb1* expression and the morphology of *tb1* mutant plants suggest that *tb1* acts both to repress the growth of axillary organs and to enable the formation of female inflorescences. The maize allele of *tb1* is expressed at twice the level of the teosinte allele, suggesting that gene regulatory changes underlie the evolutionary divergence of maize from teosinte.

Teosinte plants typically bear an elongated lateral branch at most nodes on their main stems[2]. These branches are tipped by male inflorescences (tassels) and the slender female inflorescences (ears) are borne on secondary branches in the axils of the leaves on the primary branches. In contrast, maize plants typically produce a lateral branch at only two or three of the nodes on their main stems, and these are short and tipped by ears. Previously, we demonstrated that these differences in plant architecture are governed by a small number of quantitative trait loci (QTL)[5]. One of these QTL was shown by genetic complementation testing to correspond to the *teosinte branched1* (*tb1*) locus, and it is this QTL that largely controls these differences in plant architecture[3]. In fact, the effects of *tb1* alone are sufficiently large that, when the maize chromosome segment carrying this gene is transferred into teosinte, it fully converts teosinte to maize plant architecture.

The *tb1* mutant of maize causes a complete loss of apical dominance, allowing the unrestrained outgrowth of axillary buds[3,6]. At the basal nodes of the main stem, plants homozygous for the reference allele (*tb1-ref*) produce a profusion of tillers (basal branches) where wild-type plants typically produce few, if any, tillers because the outgrowth of most of their axillary buds is completely arrested (Fig. 1a, c). At some upper nodes, mutant plants bear long lateral branches tipped by tassels where wild-type maize plants have only short branches tipped by ears (Fig. 1b, d). Because both tillers and upper lateral branches arise from axillary meristems, in a general sense, *tb1* controls the fate of axillary meristems, resulting in different outcomes depending on the position of the meristem along the main culm. At lower nodes, *tb1* controls fully arrested buds versus tillers, and at upper nodes, it controls ear shoots versus elongated branches.

To characterize further the mutant phenotype, we studied a population of 135 F_2 plants segregating for *tb1-ref* in the background of the maize inbred line A158 (Table 1). The conversion of the inflorescences terminating the lateral branches from female (wild-type) to male (mutant) was fully recessive. However, the effect of *tb1-ref* on the number of tillers was semidominant, which is consistent with a previous report[7]. Semidominance was also shown

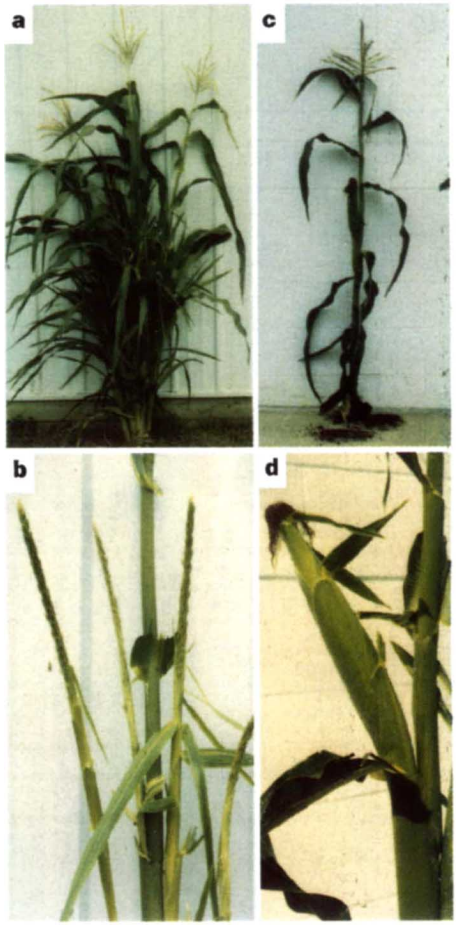

Figure 1 Plants and inflorescences of wild-type and *tb1-ref* mutant maize. **a**, **b**, *teosinte branched1* mutant. **c**, **d**, Wild-type maize (Inbred A158).

in our population for the degree of tiller growth, the number of ear shoots and lateral branch length.

Comparison of mutant and wild-type plants shows that *tb1* increases the number of primary lateral branches and tillers on the plants by allowing the outgrowth of buds rather than by increasing the number of axillary buds formed on the main stalk. First, *tb1* does not affect the total number of nodes (each potentially bearing a single axillary bud) in the main culm (Table 1). Second, *tb1* does not extend branching up the main culm, that is, the node (position) of the uppermost lateral branch in *tb1* mutants is the same as that in wild-type plants. Third, the extra tillers in *tb1* mutants result from the primary tillers each having one or two secondary tillers.

tb1 also affects the development of secondary lateral branches. In teosinte, these branches bear ears; in modern maize, these branches usually do not develop. *tb1-ref* plants typically form sterile, tassel-like inflorescences on many secondary lateral branches, although rarely they can produce small and poorly formed ears[8]. The fact that *tb1-ref* plants, unlike teosinte, do not normally produce ears on secondary branches highlights an important point. *tb1-ref* mutant plants have a teosinte-like phenotype only in their primary lateral branches and not in the secondary ones. Thus, *tb1* is not a truly atavistic mutation.

To isolate the *tb1* gene, we used the *Mutator* (*Mu*) transposable element system[9]. Homozygous *tb1-ref* plants were crossed to an active *Mu* stock, and 26,000 F_1 plants were grown. Among these, three new *tb1* mutants (*tb1-mum1*, *tb1-mum2* and *tb1-mum3*) were observed. Each new mutant was crossed to maize inbred A632, and

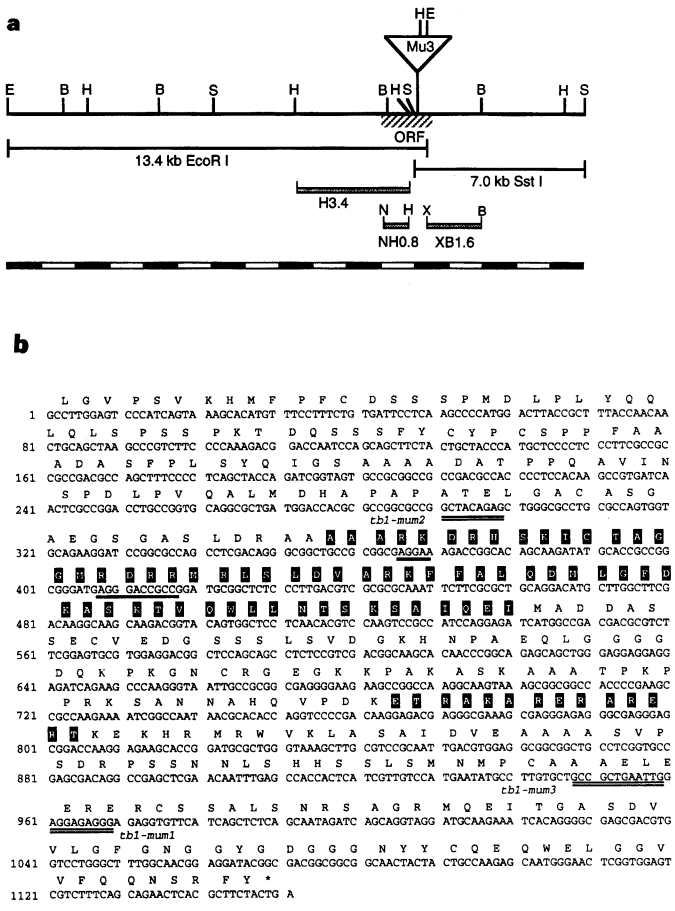

Figure 2 Structure of *teosinte branched1*. **a**, Restriction endonuclease maps of overlapping genomic λ clones (E13.4 and S7.0) of *tb1*. Insertion site of the *Mu3* element in the *tb1* open reading frame (ORF, hatched box) is shown. Three probes (H3.4, NH0.8 and XB1.6) used in Southern and northern blot analyses are shown. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; S, *Sst*I; and X, *Xba*I. Not all N and X sites were mapped. Scale in 1 kb segments. **b**, Nucleic-acid and deduced amino-acid sequence of the *tb1* cDNA clone. Two domains conserved between *tb1*, *cycloidea* and *Arabidopsis* ESTs are highlighted. Putative bipartite nuclear localization signal is underlined. Sequences duplicated by the *Mu* insertions are double underlined.

Table 1 Morphometric comparison of mutant and wild-type alleles of *tb1*

Trait	Genotypes *tb1/tb1*	*Tb1/tb1*	*Tb1/Tb1*
Tiller number per plant	8.00	1.41	0.24
Tiller growth as % of length of main stem	–	45.00	7.00
Number of visible axillary shoots	10.50	3.59	1.93
Length of uppermost lateral branch (cm)	52.51	24.06	16.21
Number of ear shoots	–	2.25	1.69
Node of the uppermost axillary shoot	10.85	10.85	10.52
Number of nodes on main stem	17.13	16.85	16.31
Number of plants measured	40	66	29

Bold underlining indicates the results of Scheffe F-tests such that a continuous line under adjacent values in a row indicates statistically equivalent values and a broken line between two values indicates statistically different values ($P = 0.05$). Plants were genotyped by Southern blot analysis with the H3.4 probe (Fig. 2a).

14 progeny from each of these crosses were grown to small seedlings and then used for DNA extraction. Southern blot analysis using marker loci that closely flank *tb1* was used to discriminate progeny that possessed the *tb1-ref* versus the new *Mu* alleles[10]. We also screened the progeny by Southern blot analysis with *Mu* element probes and thereby identified a *Mu3* element that cosegregated with *tb1-mum1*. We examined an additional 156 progeny from this family and found that this *Mu3* element was associated with the new mutant allele in 169 of the 170 plants. We attribute the single exception to either pollen contamination or a germinal reversion.

Overlapping genomic restriction fragments carrying the cosegregating *Mu3* element were cloned into λ vectors (Fig. 2a). Subclones of these were used for Southern blot analyses of the 20 full sibs of each of the three new mutant plants, showing that each of these mutants has an insertion into this region of the genome that did not exist in their sibs and the progenitor stocks (Fig. 3a). These observations provide the crucial evidence that we have newly tagged *tb1*. We sequenced portions of the λ clones and designed oligonucleotide primers to use in conjucntion with a *Mu*-specific primer to amplify (using polymerase chain reaction (PCR)) the insertion fragments for each of the three *Mu* alleles. DNA sequence analysis of these fragments enabled us to identify the *Mu* insertion point of each allele (Fig. 2b).

Northern blot analysis with RNA from various tissues and both mutant and wild-type plants revealed a 1.5-kb message in wild-type maize axillary inflorescence (ear) primordia, immature internodes below these primordia, and immature husks surrounding these primordia (Fig. 3b). The *tb1-ref* allele has a larger message (about 2.5 kb) that was expressed in the same tissue types as the wild-type allele. The *tb1-ref* message is less intense than that of the wild-type allele, suggesting either that it is expressed at a lower level or its message is less stable. The *tb-mum1* allele has no apparent message, suggesting that the *Mu* insertion may interfere with message stability. Teosinte produces the same size message as maize, indicating that maize evolution did not involve the creation or loss of *tb1*.

We used the 0.8-kb *Nco*I–*Hin*dIII fragment to screen a cDNA library derived from immature ear. We obtained a single clone with a 1,306 bp insert, excluding the poly(A)tail. The sequence of this clone (Fig. 2b) agreed fully with the genomic clone of maize inbred A619, but differed from the W22 genomic clone by a few polymorphisms that represent allelic differences. The genomic and cDNA sequences are fully collinear without any evidence for introns. This clone would appear to be nearly full length. Further experiments are needed to map the 5′ end of the gene.

We searched the GenBank database and found that *tb1* shares two short regions of homology with the *cycloidea* gene of snapdragon[4], and three *Arabidopsis* expressed sequence tags (ESTs: R29994, R30409, T45419) (Fig. 2b). First, a 62-amino-acid region is found in all sequences and a second shorter reigon is shared with one *Arabidopsis* EST, *cycloidea* and *tb1*. The conservation of these domains between maize, snapdragon and *Arabidopsis* indicates that they are important to protein function. The 62-amino-acid reigon of homology contains a putative nuclear localizaiton signal, which suggests that these proteins may play a role in the regulation of transcription[4].

There are several parallels between *cycloidea* and *tb1*. First, both mutants affect axillary structures, either flowers (*cycloidea*) or branches (*tb1*). Second, both genes have been proposed to function as repressors of organ growth[3,4]. Third, both genes affect the growth of petals and stamens, organs whose development is regulated by the B class of floral organ identity genes[11]. *Cyc*$^+$ arrests the growth of both the dorsal petal and stamen of the snapdragon flower[4]. In the

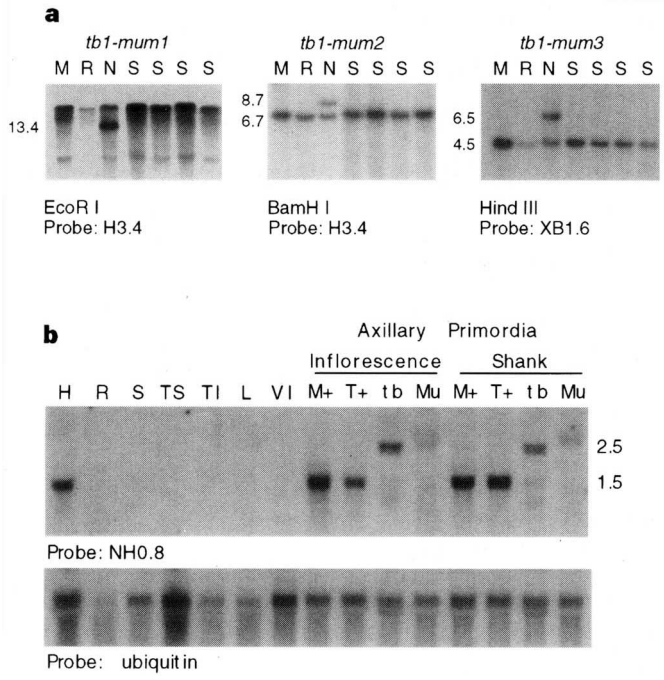

Figure 3 a, Southern blot analyses. *tb1-mum* alleles each contain an insertion in *tb1* as compared to the parental *tb1-ref* and *Mu* stocks, and 20 (4 shown) full sibs of each mutant plant. The *Mu3* element in the *tb-mum1* allele possesses an *Eco*RI site, so this insertion created the 13.4 kb *Eco*RI fragment. The *Mu* insertions in the other two *tb1-mum* alleles increased the sizes of existing fragments by about 2 kb. Abbreviations: *Mu* stock, M; new *Mu* mutant plant from the tagging population, N; *tb1-ref* stock, R; full sibs of the new *Mu* mutant plant, S. **b**, Northern blot analyses. RNAs from inbred W22: ear husk, H; root, R; dark grown seedlings, S; immature tassel spikelets, TS; immature tassel rachis internodes, TI; immature leaves, L; and immature vegetative internodes of the main stem, VI. Additional RNAs from axillary inflorescence and shank primordia of plants homozygous for alleles: *Tb1 + Maize*, M+; *Tb1 + teosinte*, T+; *tb-ref*, tb; *tb1-mum1*, Mu.

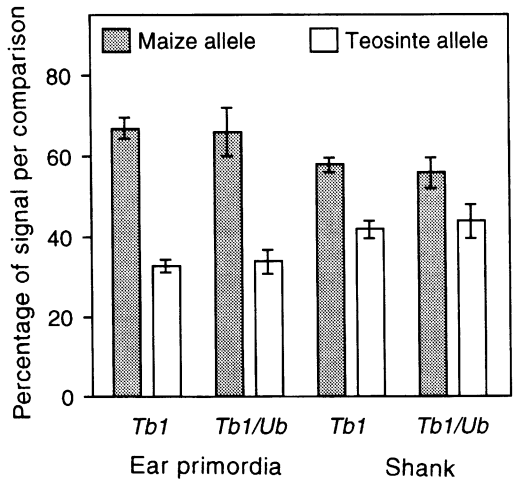

Figure 4 *tb1* message accumulation for ear primordia and immature shank of the maize (*Tb1 + Maize*) and teosinte (*Tb1 + teosinte*) alleles in inbred W22 genetic background. Data are presented both as the amount of *Tb1* message per 10 μg of total cellular RNA and as the amount relative to *Ubiquitin* (*Ub*) message level as determined by phosphor imager readings. Probability (*P*) under the null hypothesis of no difference in message level of the maize and teosinte alleles was less than 0.01 in each case except for *Tb1/Ub* for shank ($P < 0.05$); standard error bars as shown.

ear where *tb1* is expressed, growth of the lodicules (petals) and stamens is arrested.

A previous proposal[3] that *tb1* acts as a repressor of axillary meristem growth is consistent with our expression data in that the axillary organs in which *tb1* is expressed are typically reduced in size. For example, the husks of wild-type maize have reduced leaf blades and the axillary branch internodes of wild-type maize are shorter. Thus, *tb1* may act as a repressor of the growth of those organs in which the gene is expressed. *tb1* also regulates the sex of the inflorescences terminating the lateral branches and is required for the normal formation of ears. This may indicate that *tb1* has functions in addition to that of repressing organ growth.

Previously, we proposed that maize and teosinte would both carry functional alleles of *tb1* but ones whose expression is differentially regulated[3]. A higher level of expression for the maize allele is expected in correspondence with its shorter (more repressed) axillary branches. To test this, we quantified *tb1* mRNA levels for ear primordia from 12 plants of maize inbred W22 (carrying *Tb1 + Maize*) and 12 plants of this same inbred but with *Tb1 + teosinte* (Fig. 4). The *Tb1 + Maize* sample had a mean band intensity twice that of *Tb1 + teosinte* ($P < 0.01$), consistent with this expectation. The message level of the maize allele in immature shanks is also greater than that of the teosinte allele. The relative mRNA level for a wider sample of maize and teosinte alleles needs to be analysed to assure the generality of these results.

In view of data presented here, the following model for *tb1* in maize evolution is proposed[3]. In teosinte, *tb1* is functional and is normally expressed in the secondary axillary meristems where it controls their conversion into ear shoots (including the small ear, the single husk that surrounds this ear and the single short internode that subtends it). *tb1* is not normally expressed in the primary axillary meristems of teosinte so that these are able to develop into elongated tassel-tipped branches. During the domestication of maize, humans selected an allelic variant of *tb1* that is expressed in primary axillary meristems (and probably has a high level of expression) such that these form ear shoots rather than elongated tassel-tipped branches. Thus, evolution has not proceeded by a loss/gain or change in *tb1* function, but by an alteration in gene regulation. Consistent with this view, a preliminary comparison of partial amino-acid sequences of maize and teosinte alleles revealed no fixed amino-acid differences between them, suggesting that a change in protein function has not occurred (see Supplementary Information).

The cloning of *tb1* provides a new tool not only for understanding maize evolution but also for the study of plant development. *tb1* is involved in controlling apical dominance, inflorescence development and sex determination, processes of broad interest in plant developmental biology. It may also be possible to engineer *tb1* or *tb1*-like genes to provide increased apical dominance in other crops. Using the tools of molecular genetics, it will be possible to determine whether *tb1*-like genes have played a role in the domestication of other crops, such as sunflower, that show increased apical

dominance relative to their wild ancestors[1]. Comparative QTL mapping[12] suggests that this is not an unlikely prospect. □

Methods

Plant materials. The W22 line carrying *Tbl* + *teosinte* was constructed as previously described[3] except with six generations of backcrossing. The *tb1-ref* stock was provided by C. Burnham and the *Mu* stocks by V. Chandler. The *Mu* tagging population, the F_2 population segregating for *tb1-ref*, and plants for quantification of *tb1* message levels were grown at the University of Minnesota Agricultural Experiment Station in St Paul during the summers of 1994, 1995 and 1996, respectively.

Nucleic acid analysis. DNA extraction and Southern hybridizations were done as previously described[5]. Molecular marker loci closely flanking *tb1* were bcd1072, npi615, umc107 and umc140. Genomic DNA restriction fragments carrying the cosegregating *Mu3* element were cloned into either λ-Dash or λ-ZAP (Stratagene), following manufacturer's instructions. The cDNA library, constructed in λ-ZAP Express (Stratagene) from RNA isolated from immature ears of Pioneer line AP9, was provided by S. Briggs and T. Helentjaris (Pioneer Hi-Bred). Total cellular RNA was isolated using TRI reagent (Molecular Research Center) according to manufacturer's instructions. Northern blot analyses were performed using 10 μg total RNA per lane as described elsewhere[13]. RNA loading was quantified by stripping the membranes and reprobing with a 0.65 kb *PstI–SacI* subclone of a maize *Ubiquitin* cDNA probe[14]. *tb1* and *Ubiquitin* expression for the W22 : *Tbl* + *Maize* and W22 : *Tbl* + *teosinte* samples was determined by scanning northern blots with a Molecular Dynamics Storm (Phosphor) imager. Ear primordia used in the quantitative analyses (Fig. 4) were staged by length, and there was no difference between the mean lengths of the maize (29.3 mm ± 1.8) and teosinte (29.4 mm ± 1.6) genotypes.

PCR was performed using PCR Supermix (Life Technologies) with 35 cycles of 1 min at 95 °C, 1 min at 58 °C and 3 min at 72 °C followed by 10 min at 72 °C. DNA substrates were from plants carrying the three *tb1-mum* alleles. Each reaction included the *Mu* primer (CCAACGCCAWSGCCTCCATTTCGTCGA ATCC) and one of the following *tb1*-specific primers (JD72: GGTCACTTGT AGCCAATAGC, JD76: CCTACCTGCTGATCTATTGC, JD83: GGTCAGATA GTAAGTTGTGC, JD88: TAATACATCATCATGCGAT, JD96: TCCCATCAGT AAAGCACATG). Primers JD72 and JD76 were also used to amplify a 1,500 bp portion of the *tb1* gene from maize inbred A619. PCR products were cloned using the TA Cloning Kit version D (Invitrogen) following manufacturer's recommendations. DNA sequencing was done by the Advanced Genetics Analysis Center (University of Minnesota).

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Supplementary Information is available on *Nature's* World-Wide Web site (http://www.nature.com) or as paper copy from Mary Sheehan at the London editorial office of *Nature*.

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A common precursor for primitive erythropoiesis and definitive haematopoiesis

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The generation of blood cells, haematopoiesis, in the mouse embryo begins with the development of primitive nucleated erythroid cells in the yolk sac followed by the appearance of precursors for multiple definitive haematopoietic lineages[1–4]. The later developing lineages arise from multipotential stem cells[5,6], but the relationship of primitive erythroid cells to these other haematopoietic populations is unknown. Using an *in vitro* embryonic stem (ES) cell differentiation system[7], we show that primitive erythrocytes and other haematopoietic lineages arise from a common multipotential precursor that develops within embryoid bodies generated from differentiated ES cells. In response to vascular endothelial growth factor and c-kit ligand these precursors give rise to colonies containing immature cells (blasts) expressing marker genes characteristic of haematopoietic precursors. Many blast colonies also expressed βH1 and β major globins but not Brachyury, a mesodermal marker. Kinetic analysis demonstrated that the blast colony-forming cells represent a transient population, preceding the establishment of the primitive erythroid and other lineage-restricted precursors. This precursor population may represent the earliest stage of embryonic haematopoietic commitment.

Previous studies have demonstrated that the primitive erythroid lineage is established within developing embryoid bodies (EBs) by day 4 of differentiation[8]. To identify earlier precursor populations, the current series of experiments focused specifically on EBs differentiated for shorter periods of time. When cells from EBs that had been differentiated for 3 to 3.5 days were plated in methyl cellulose cultures in the presence of vascular endothelial growth factor (VEGF) and c-kit ligand (KL), colonies consisting of readily identifiable cells with an undifferentiated or blast morphology developed within 2 to 3 days (Fig. 1a, b). Secondary EBs, which were also present in these cultures, were distinguished from the blast cell colonies by the fact that they are made up of densely packed cells (Fig. 1a). Additionally, the cells within the EBs tend to have a slightly higher cytoplasm to nucleus ratio than those found in the blast colonies (Fig. 1b).

When individual blast cell colonies (4–6 days old) were picked and replated into secondary methyl cellulose cultures containing a broad spectrum of cytokines, colonies of many different haematopoietic lineages developed. The most striking pattern observed was that cultures containing colonies of primitive erythroid cells (Ery^p; Fig. 1c, d) also contained colonies of other haematopoietic lineages including definitive erythroid (Ery^d; Fig. 1e, f), multilineage (Fig. 1g, h) and myeloid (not shown). The erythroid populations were identified based on the fact that Ery^p cells are large, nucleated and express βH1 globin whereas the Ery^d cells are smaller, enucleated and express little if any embryonic globin[9,10]. When scoring cultures, individual Ery^p and Ery^d colonies were distinguished by the size of

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