Biochem. J. (1990) 268, 641–648 (Printed in Great Britain)

Isolation and characterization of the mouse liver/bone/kidney-type alkaline phosphatase gene

Mineko TERAO, Michela STUDER, Maurizio GIANNÍ and Enrico GARATTINI*
Molecular Biology Unit, Centro Daniela e Catullo Borgomainerio, Istituto di Ricerche Farmacologiche 'Mario Negri', via Eritrea 62, 20157 Milano, Italy

The gene coding for the mouse alkaline phosphatase expressed in liver, bone, kidney and placenta (liver/bone/kidney-type alkaline phosphatase, L/B/K-ALP) was isolated and characterized. This gene consists of 12 exons and it is at least 49 kb long. The first two exons are separated by a long intron which is at least 32 kb in size, whereas the other exons span within the remaining 17 kb. Primer extension and S1-nuclease mapping analyses with placental mRNA demonstrate a single major transcription start site, which is preceded by a G+C-rich region containing a TATA-like sequence and three copies of the consensus binding site for the transcription factor Sp1. Transfection experiments using two different reporter genes show that the 5'-flanking region of the gene is active as a promoter in undifferentiated F9 teratocarcinoma cells, but not in 3T3 fibroblasts, consistent with the L/B/K-ALP mRNA level in the two cell lines. As expected from the sequence similarity at the cDNA level, the structural organization of the mouse gene is similar to that of the human and rat L/B/K-ALP genes, suggesting that they all derive from a single ancestral gene.

INTRODUCTION

Mammalian alkaline phosphatases (ALPs) [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] are a family of membrane-bound glycoproteins capable of hydrolysing various organic monophosphate esters *in vitro*. ALP enzymic activity is found both in normal tissues and in various tumours and tumour cell lines such as teratocarcinomas (Bernstine *et al.*, 1973; Hass *et al.*, 1979), choriocarcinomas (Hamilton *et al.*, 1979) and osteosarcomas (Benham *et al.*, 1981). At least two isoenzymes coded for by distinct genetic loci have been proposed in the mouse (Goldstein *et al.*, 1980; Wilcox, 1983), and additional isoenzymes are found in humans and higher primates (McKenna *et al.*, 1979; Sergeant & Stinson, 1979). In the adult mouse, one of the ALP isoenzymes is predominantly expressed in liver, bone, kidney and placenta (L/B/K-ALP), whereas the expression of the other form is restricted to the intestine (intestinal ALP) (McComb *et al.*, 1979). ALP activity is also found in the early embryos at various stages of development (Chiquoine, 1954). Particularly high levels of ALP expression are observed in primordial germ cells, whose migration towards the gonads can be traced by using ALP activity as a marker (Mintz & Russel, 1957). The physiological role of the various forms of ALP in the adult organism as well as during the development of the embryo is still unknown, although various theories have been proposed (McComb *et al.*, 1979).

The cDNA coding for mouse placental ALP was recently cloned and its deduced amino acid sequence showed 90% similarity to that of the human L/B/K-type ALP (Weiss *et al.*, 1986; Terao & Mintz, 1987), but only 55% identity with the human placental as well as intestinal isoenzymes (Kam *et al.*, 1985; Ovitt *et al.*, 1986; Henthorn *et al.*, 1986; Millan, 1986; Berger *et al.*, 1987; Henthorn *et al.*, 1987). The L/B/K-ALP gene was mapped to chromosome 4 in the mouse (Terao *et al.*, 1988) and to chromosome 1 in humans (Swallow *et al.*, 1986). This is consistent with the fact that the distal portion of mouse chromosome 4 and the short arm of human chromosome 1 carry the largest segments of conserved linkage between mouse and human (Lalley *et al.*, 1978; Nadeau & Taylor, 1984; Lalley & McKusick, 1985; Marth *et al.*, 1986). Recently the structure of the human L/B/K-type, intestinal and placental ALP gene loci have also been elucidated. The L/B/K-type ALP is structurally distinct from that of the placental and intestinal ALP genes, which are closely related and are mapped to chromosome 2 (Weiss *et al.*, 1988; Henthorn *et al.*, 1988; Knoll *et al.*, 1988). The rat L/B/K-type ALP cDNA and gene were also isolated; however, their chromosome location is not known (Misumi *et al.*, 1988; Toh *et al.*, 1989).

As a first step in understanding the function and the mechanisms regulating the expression of L/B/K-ALP in the various tissues of the adult mouse, and of the mouse embryo during the development, we have undertaken the isolation and structural characterization of the gene coding for this enzyme. As expected on the basis of the similarity at the cDNA level (Weiss *et al.*, 1986; Terao & Mintz, 1987; Misumi *et al.*, 1988), the structure of the mouse L/B/K-ALP is very similar to its human and rat counterparts (Weiss *et al.*, 1988; Toh *et al.*, 1989) in terms of its length, organization and putative regulatory elements. The function of the L/B/K-ALP gene promoter was also examined in an embryonal carcinoma and a fibroblast-derived cell line showing high and low levels of ALP activity respectively. The results of these experiments suggest the presence of elements capable of conferring cell specificity of transcription in the promoter region.

EXPERIMENTAL

Cell lines

F9 teratocarcinoma cells (kindly given by Dr. B. Terrana, Sclavo Laboratories, Siena, Italy) and the fibroblastic cell line 3T3 (obtained from the American Type Culture Collection, Rockville, MD, U.S.A.) were grown in Dulbecco's minimal essential medium (Gibco Laboratories, Grand Island, NY, U.S.A.).

Abbreviations: ALP, alkaline phosphatase; L/B/K-ALP, liver/bone/kidney-type alkaline phosphatase; hGH, human growth hormone; CAT, chloramphenicol acetyltransferase; S1, S1 nuclease.

* To whom correspondence and reprint requests should be sent.

These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases.

Isolation and characterization of ALP genomic clones

Two libraries in the phage vector EMBL3A obtained by endonuclease-*Mbo*I partial digestion of genomic DNA derived from liver and T-lymphocytes of C57Bl/6J mice were kindly given by Dr. Y. Uematsu (Roche Institute of Immunology, Basel, Switzerland) and by Dr. L. Mori (Istituto di Farmacologia, Università degli Studi di Milano, Italy) respectively. A genomic library was constructed in the phage vector lambda Dash (Stratagene, San Diego, CA, U.S.A.) as follows. Thymus DNA derived from C57Bl/6J mice was digested to completion with *Bam*HI, and fractionated on a 0.8%-agarose gel (Maniatis *et al.*, 1982). Fractions containing DNA hybridizing with the full-length mouse placental cDNA (Terao & Mintz, 1987) were used for packaging *in vitro* with GigaPack (Stratagene, San Diego, CA, U.S.A.), and the resultant recombinant phages were amplified in *Escherichia coli* LE392(P2).

Phages containing the mouse L/B/K-ALP gene were isolated from genomic libraries by plaque hybridization *in situ* (Maniatis *et al.*, 1982) using either different mouse placental cDNA fragments (for clones 81, 51, 21, 8K, 6K, 86 and 61) or a genomic fragment (for clone N1) as probes (see also legend to Fig. 1 below), labelled with ^{32}P by the random-priming method (Feinberg & Vogelstein, 1983).

Restriction-endonuclease mapping of the inserts and preparation of DNA were carried out according to standard procedures (Maniatis *et al.*, 1982). DNA blotting analysis was performed on nylon membranes (GeneScreen Plus; NEN Research Products, Boston, MA, U.S.A.) according to the manufacturer's instructions. The position of exons was determined by DNA blotting analysis using ^{32}P-labelled cDNA fragments or specific oligonucleotides as probes, and by DNA sequence analysis. When oligonucleotides were used for DNA blotting analysis, probes were labelled at their 5'-end with ^{32}P with polynucleotide kinase (Maniatis *et al.*, 1982), and hybridization and washing were performed as previously described (Wood *et al.*, 1985).

DNA sequence analysis

DNA fragments were subcloned into the plasmid vector pUC18 or Bluescript (Stratagene) and sequenced by the dideoxynucleotide-chain-termination method (Sanger *et al.*, 1977), using double-stranded DNA as templates and Sequenase (United States Biochemical Co., Cleveland, OH, U.S.A.) according to the manufacturer's instructions. Appropriate oligodeoxynucleotide primers were prepared with a Beckman (Palo Alto, CA, U.S.A.) Sys-200 oligodeoxynucleotide synthesizer. Sequence analysis of the promoter region was performed after generation of nested deletions in both orientations (Henikoff, 1984). Computer analysis of the DNA sequences was performed using the Microgenie sequence analysis system (Beckman).

Primer extension and S1-nuclease (S1) mapping analysis

Total RNA was prepared from mouse placenta, and polyadenylated RNA was selected according to standard procedures (Maniatis *et al.*, 1982). Primer-extension analysis was carried out with an 18-base synthetic oligonucleotide complementary to nucleotides 337–354 of mouse placental cDNA (Terao & Mintz, 1987) labelled at its 5'-end with ^{32}P by phage-T_4 polynucleotide kinase (Maniatis *et al.*, 1982). The primer was mixed with RNA and precipitated with ethanol. The pellet was resuspended in 10 μl of extension buffer containing 0.05 M-Tris (pH 8.3)/0.15 M-KCl/0.5 mM-EDTA/7 mM-$MgCl_2$/1 mM-dithiothreitol/actinomycin D (25 mg/ml; Sigma), heated at 65 °C for 2 min and cooled on ice for 5 min. Reverse transcription was started by addition of 1 μl of 5 mM-deoxynucleotide triphosphates and 7 units of avian-myeloblastosis-virus reverse transcriptase (International Biotechnologies, New Haven, CT, U.S.A.). After incubation at 42 °C for 90 min, the reaction was stopped by the addition of 2 μl of 0.5 M-NaOH, heated at 100 °C for 3 min and neutralized with 2 μl of 0.5 M-HCl and 2 μl of 1 M-Tris, pH 7.5. After ethanol precipitation, samples were resuspended in 95% formamide/0.02 M-EDTA/0.05% Bromophenol Blue/0.05% Xylene Cyanol FF, and electrophoresed on a 6%-(w/v)-polyacrylamide/7 M-urea gel.

The probe used for S1 mapping analysis was a 165-nucleotide *Ava*I–*Pst*I fragment (from nucleotide −88 to nucleotide 77 in Fig. 4 below), labelled with ^{32}P at the 5'-end of the *Pst*I site by phage-T_4 polynucleotide kinase (Maniatis *et al.*, 1982). Hybridization of the probe to the polyadenylated RNA was done overnight in a buffer containing 0.04 M-Pipes (pH 6.4)/0.001 M-EDTA/0.4 M-NaCl/80% (v/v) formamide at 55 °C, followed by S1 nuclease digestion (Berk & Sharp, 1977). The S1-protected DNA fragments were analysed on a 12%-polyacrylamide/7 M-urea sequencing gel in parallel with sequencing ladders of the probe. The sequencing ladder was obtained by chemical degradation of the radiolabelled probe with base-specific reagents (Maxam & Gilbert, 1980).

Plasmids used for transfection experiments

The plasmid p0gh, containing the promoter-less human-growth-hormone (hGH) gene, and plasmid pTKgh containing the same gene under the control of the viral thymidine kinase promoter are commercially available (Nichols Institute, San Juan Capistrano, CA, U.S.A.). ALPgh was constructed as follows. The 2.6 kb *Eco*RI fragment containing exon 1 and the flanking region of ALP gene was isolated from lambda clone 81 (see Fig. 1 below) and subcloned in Bluescript. This plasmid was further digested with *Pst*I, and the resulting 1.9 kb fragment was re-subcloned in Bluescript (ALP-Blu). ALP-Blu was digested with *Xba*I and *Eco*RV to release the insert containing 77 bp of the first exon as well as 1.8 kb of 5'-flanking region and 13 bp of the vector polylinker. This fragment was inserted in front of the hGH gene in p0gh. For this purpose p0gh was digested with *Bam*HI, blunt-ended and cleaved with *Xba*I.

The plasmid P106, kindly given by Dr. M. Atchison (The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA, U.S.A.) contains the gene for bacterial chloramphenicol acetyltransferase (CAT) downstream of a polylinker region (Atchison *et al.*, 1989). The plasmid pSV_2CAT is commercially available (Pharmacia, Uppsala, Sweden) and contains the CAT gene under the control of the T-antigen promoter-enhancer of simian virus 40. ALP-Blu was digested with *Eco*RV and *Xba*I, and the resulting 1.9 kb fragment was inserted in *Xba*I–*Sma*I-digested P106 to produce ALPCAT. In this construct the putative ALP promoter region is in the correct orientation with respect to the CAT gene. rALPCAT was constructed by cleaving ALP-Blu with *Eco*RV and *Hin*dIII, and by inserting the released fragment in P106, previously cut with *Sma*I and *Hin*dIII. In this construct, the putative ALP promoter is inserted in the opposite orientation with respect to the CAT gene.

Transfections and assays

Cells were transfected with circular plasmid DNA (20 μg/10 cm dish) using the calcium phosphate co-precipitation method (Graham & Van Der Eb, 1973). In the case of ALPgh, as well as appropriate control plasmids, conditioned medium was collected 48 h after transfection and assayed for hGH immunoreactivity with a commercially available radioimmunoassay kit (Nichols Institute). In the case of ALPCAT, rALPCAT and appropriate control plasmids, cells were harvested 48 h after transfection and assayed for CAT activity (Gorman *et al.*, 1982). Protein assays were performed by the method of Bradford (1976).

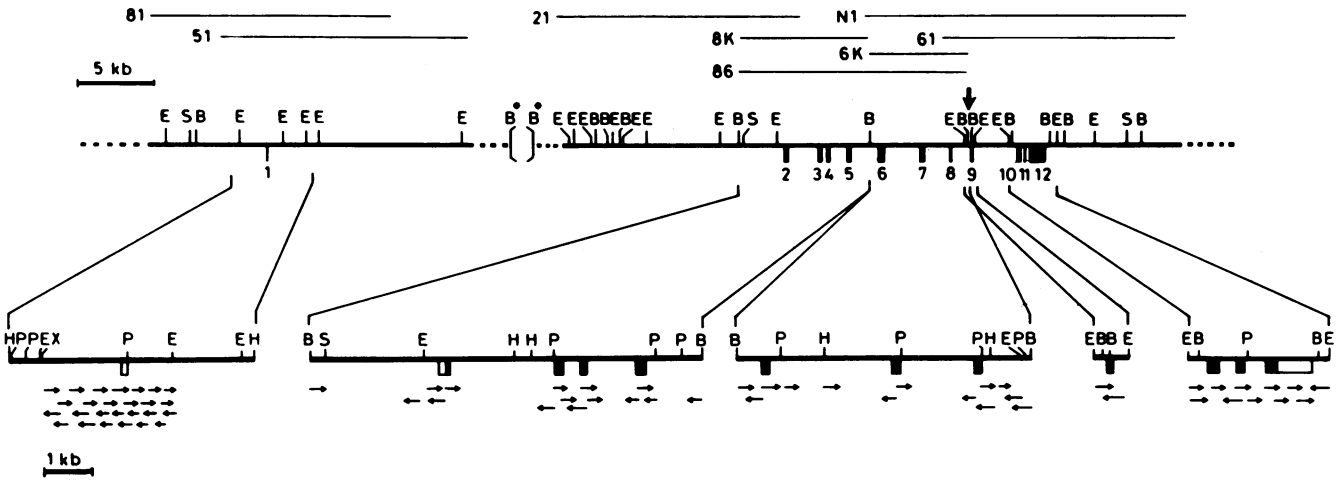

Fig. 1. Organization and sequence strategy for the mouse L/B/K-ALP gene locus

Thick solid lines represent the L/B/K-ALP locus, with transcription proceeding from left to right. Thin solid lines, shown at the top of the Figure, represent inserts of recombinant phages and are labelled with numbers. Clones 8K, 6K and 86 were isolated from the *Bam*HI–lambda Dash library using mouse placental full-length cDNA as a probe. All other clones were from *Mbo*I–EMBL 3A libraries. The probe used for the isolation of clone 61 was the full-length cDNA, whereas the one used for clones 81, 51 and 21 was a 682 bp fragment representing the 5′-end of the cDNA. The probe used for the isolation of clone N1 was a *Bam*HI genomic fragment whose position is indicated by a vertical arrow in the Figure. Portions of the gene containing exons are enlarged. Exons are shown as boxes underneath the line of the gene, and are numbered from 1 to 12. In the enlarged scheme, black areas within boxes represent translated regions, whereas white areas represent untranslated regions of mouse L/B/K-ALP cDNA. Restriction-endonuclease sites are indicated by capital letters (E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I; S, *Sal*I). The position of *Bam*HI sites noted with asterisks as well as the dotted line in the middle of the gene is predicted by DNA blotting experiments using genomic subclones derived from the edges of clone 81 and 21 as probes. Brackets indicate the possibility of additional *Bam*HI fragments. The direction and length of each sequence analysis for the L/B/K-ALP gene are indicated by horizontal arrows. Sequencing was performed either using specific oligonucleotides as primers or from various restriction sites after cloning of appropriate subfragments.

RESULTS

Mouse L.B/K-ALP gene

A physical map of the mouse L/B/K-ALP gene is shown in Fig. 1. This map was obtained by restriction cleavage of lambda genomic clones isolated from three different libraries. DNA blotting analysis was performed by using mouse placental full-length cDNA as a probe and is shown in Fig. 2. After digestion with *Bam*HI, all the bands appearing in the autoradiogram are accounted for by the physical map shown in Fig. 1, except for a 0.2 kb fragment containing exon 9, which does not appear in the Figure, and a large fragment (at least 23 kb) containing exon 1, which appears only after longer exposure of the film because of its low transfer efficiency. The *Eco*RI and *Hin*dIII cleavage patterns are again consistent with the physical map of the gene, except for a 0.8 kb *Eco*RI fragment containing exon 9 that is not visible on the autoradiogram shown.

The ALP gene extends for at least 49 kb and it consists of 12 exons. The nucleotide sequence of exon–intron junctions (Table 1) conforms with the consensus sequence established for other eukaryotic genes: introns always start with GT and end with AG. At the 3′-end of the gene, exon 12 contains putative 3′-mRNA processing signals that are commonly found in other eukaryotic genes (Mount, 1982; Green, 1986); the mRNA cleavage/polyadenylation site is located 16 bases downstream of the sequence AATAAA, and 17 bases upstream of a G+T-rich region (Birnstiel *et al.*, 1985). The structure of the transcribed region of the ALP gene is exactly the same as that of the mouse placental cDNA as determined by sequence analysis. The 5′-untranslated region is contained in exon 1 and part of exon 2. The protein coding region is present within 11 exons (exons 2–12), whereas its entire 3′-untranslated region is within exon 12. Exon 1 and exon 2 are separated by a long intron whose size is at least 32 kb. In fact, the two edges of the intron 1 contained in

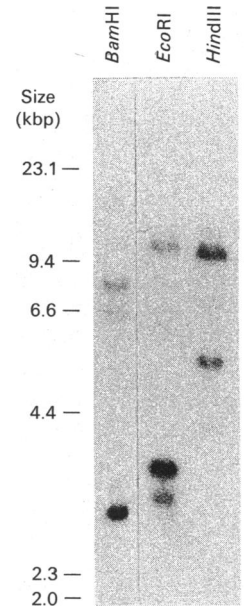

Fig. 2. DNA blotting analysis of genomic DNA

Genomic DNA obtained from C57Bl/6J mouse thymus was digested with the indicated restriction enzymes, size-fractionated on a 0.8% agarose gel, transferred to a nylon membrane and hybridized to ^{32}P-labelled mouse placental-ALP cDNA. The positions of molecular-size markers (*Hin*dIII-digested λ-phage DNA) are shown on the left-hand side.

clones 81 and 21, which are not overlapping, add up to 26 kb (Fig. 1). At least an additional 6 kb is accounted for on the basis of the results of DNA blotting analysis performed with probes

Table 1. Intron–exon organization of mouse L/B/K-ALP gene

The nucleotide sequences of intron–exon junctions were determined by the strategy shown in Fig. 1. Exon sequences are shown in capital letters and intron sequences are shown in lower-case letters. The positions of nucleotides that border the 5′- and 3′-ends of the introns are numbered above the DNA sequence with the transcription start site designated as '1' (nucleotides within introns are not numbered). Amino acids bordering the splice junctions are shown numbered with the first methionine residue of the unprocessed mouse placental ALP designated as '1'. The region of mRNA cleavage/polyadenylation at the 3′-end of exon 12 is shown at the bottom of the Table. Nucleotides not present in the cDNA that are 3′ to the polyadenylation site are shown in lower-case letters; putative 3′-mRNA processing signals, common to other eukaryotic genes, are underlined.

Exon no.	Exon length	Splice donor site	Intron no. and length	Splice acceptor site
		82		83
1	82	CCTGCAG gtaaggaaa	1 (>32 kb)	taacttctag GATCGGAA
		243		244
2	161	GTG CCA G gtaggactg	2 (2.3 kb)	gtctctgcag AG AAA GAG
		Val Pro G		lu Lys Arg
		3		5
		363		364
3	120	GGA GAT G gtgaggctg	3 (0.4 kb)	cctcccacag GT ATG GGC
		Gly Asp G		ly Met Gly
		43		45
		479		480
4	116	CTC TCC AAG gtgagccct	4 (1.0 kb)	ccacttgcag ACA TAT AAC
		Leu Ser Lys		Thr Tyr Asn
		81		84
		654		655
5	175	GAT GCT G gtgagccat	5 (1.6 kb)	ctcacttcag GG AAG TCC
		Asp Ala G		ly Lys Ser
		140		142
		830		831
6	176	GAT ATC GAC gtgagtgga	6 (2.5 kb)	tgtccctcag GTG ATC ATG
		Asp Ile Asp		Val Ile Met
		197		200
		974		975
7	144	AGA CAC AAG gtaaccaag	7 (1.6 kb)	aacccctcag CAT TCC CAC
		Arg His Lys		His Ser His
		246		249
		1044		1045
8	70	CTC TTA G gtaagtaga	8 (1.3 kb)	atgtccacag GT CTC TTT
		Leu Leu G		ly Leu Phe
		270		272
		1179		1180
9	135	GTG GAA G gtaggaagt	9 (2.7 kb)	atgtccctag GA GGC AGG
		Val Glu G		ly Gly Arg
		315		317
		1371		1372
10	192	ATC TTT G gtgagtagc	10 (0.3 kb)	ccctacgcag GT CTG GCT
		Ile Phe G		ly Leu Ala
		379		381
		1491		1492
11	120	GAT TAC G gtgagactg	11 (0.4 kb)	gacctacag CT CAC AAC
		Asp Tyr A		la His Asn
		419		421
			2459	
12	968	AAGACATTTAAATAAAACATCCCAAATATTTCTgaggccagagctgagtcttttgtggtcag		

corresponding to the 3′-end of clone 81 and to the 5′-end of clone 21 (results not shown; see also the Discussion section).

To confirm the transcription start site(s) of the mouse L/B/K-ALP gene, primer-extension and S1-mapping analyses were performed. Results are shown in Figs. 3(*a*) and 3(*b*) respectively. The major band observed after primer-extension analysis using placental polyadenylated RNA is of the expected size (354 nucleotides), whereas no bands are observed using tRNA as a control. As expected, placental RNA protects a 77-nucleotide fragment, and a minor 75-nucleotide band is also observed with longer exposure of the autoradiogram (lanes 3–5). Intestinal polyadenylated RNA or tRNA does not protect any of these bands (lanes 1 and 2 respectively). The two assays are in good agreement in determining a major transcription start site at the nucleotide indicated in Fig. 4.

The nucleotide sequence of the 5′-flanking region of the gene, as well as exon 1 and part of intron 1, is shown in Fig. 4. A promoter element, TTCATAA, similar to a TATA box (Serfling *et al.*, 1985; Dynan & Tjian, 1985), is located 25 bp upstream of the putative transcription start site. Three GGCGGG repeats at position −61, −71 and −76 are present and constitute a consensus binding site for the transcription factor Sp1 (Briggs

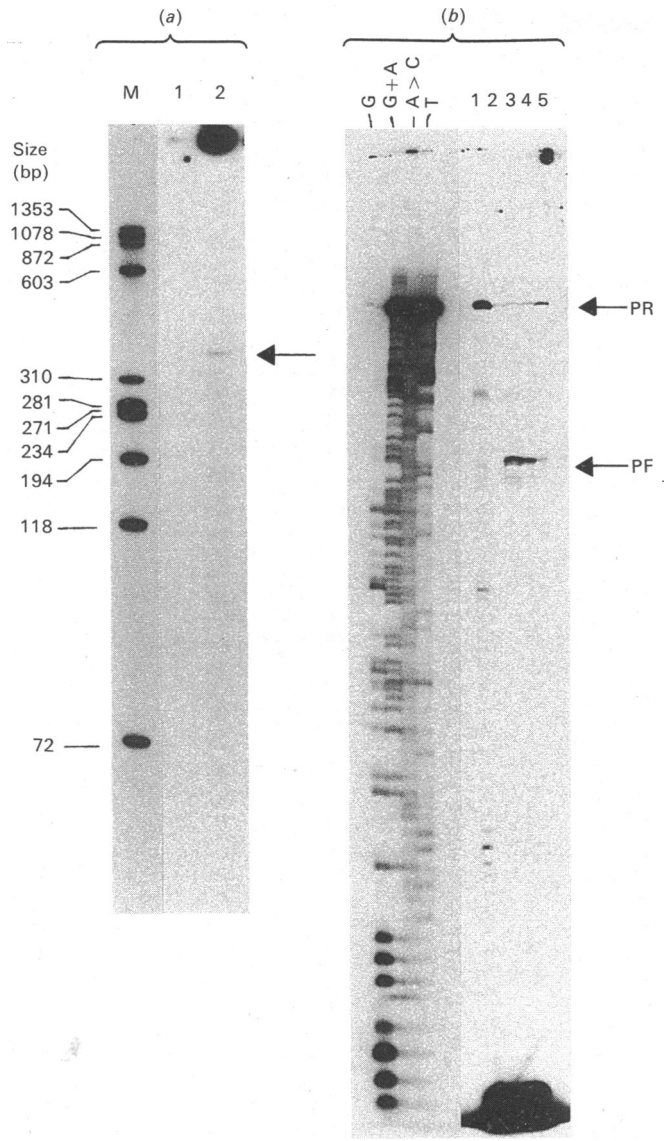

Fig. 3. Mapping of the 5′-end of mouse L/B/K-ALP mRNA

(*a*) Primer extension analysis. A 18-nucleotide oligomer complementary to nucleotides 337–354 of mouse placental ALP cDNA (Terao & Mintz, 1987) was labelled at its 5′-end and used for primer extension. The probe was annealed with 5 μg of tRNA (lane 1) or with 5 μg of mouse placental polyadenylated RNA (lane 2) and reverse-transcribed. The analysis was performed on a 6%-(w/v)-polyacrylamide/7 M-urea gel. *Hae*III-digested phage-ϕX174 DNA fragments (lane M) were used as size markers. The specific extended product is indicated with an arrow. (*b*) S1 protection analysis. S1-protected fragments (right side) were analysed on a 12%-polyacrylamide/urea sequencing gel in parallel with the sequence ladder of the probe (left side). The amounts of RNA of all the samples were adjusted to 30 μg by addition of tRNA; lane 1, S1-treated tRNA alone; lane 2, 10 μg of S1-treated intestinal polyadenylated RNA; lanes 3–5, 10, 5 and 2 μg each of S1-treated placental polyadenylated RNA respectively. The position of protected fragments (PF) and that of the reannealed probe (PR) is indicated by arrows.

et al., 1986; Kadonaga *et al.*, 1986). A perfect direct repeat consisting of 14 bp is present from position −54 to position −82, and several other imperfect 7 bp repeats are present throughout the sequenced region. A 9 bp inverted repeat is positioned between nucleotide −1184 and nucleotide −1205. The G/C content of the first 200 bp upstream to the putative capping site is 78%.

L/B/K-ALP gene expression and functional characterization of its 5′-flanking region in F9 teratocarcinoma cells and 3T3 fibroblasts

Experiments on the promoter activity of the 5′-flanking region of mouse L/B/K-ALP gene were performed using mouse F9 teratocarcinoma and 3T3 fibroblasts as recipient cells. The undifferentiated F9 cell line was chosen because it expresses high ALP activity (Bird, 1986), whereas 3T3 cells contain negligible amounts of the enzyme (about 1% in respect to F9; results not shown). Fig. 5 shows that there is a correlation between the level of enzymic activity and the steady-state level of the L/B/K-ALP transcript in the two cell lines. RNA blotting analysis, using total cellular RNA and the mouse placental cDNA as a probe, reveals a specific band of about 2500 nucleotides in F9, as expected, whereas no such band is observed in 3T3.

To determine the promoter activity of the 5′-flanking region of the mouse L/B/K-ALP gene, a 1.9 kb fragment containing this region was fused to CAT or hGH reporter genes and used to transfect F9 teratocarcinoma cells and 3T3 fibroblasts. The results of these experiments are shown in Table 2. When the ALP promoter is placed in front of the CAT gene (ALPCAT), significant elevation (about 7-fold) of CAT activity is observed in transfected F9 cells in comparison with the same construct lacking a functional promoter (p106). When the ALP promoter is placed in front of the CAT gene in the reverse orientation (rALPCAT), the level of expression is lower, although it is still above the background level (about 3-fold). On the other hand, in 3T3 cells the stimulation of CAT-gene expression due to the presence of the ALP promoter is only 2-fold. In both cell lines the simian-virus-40 promoter-enhancer is active, albeit at different levels.

The same qualitative results obtained with the CAT constructs were observed when hGH was used as a reporter gene. In F9 cells the promoter of the mouse L/B/K-ALP gene stimulates the secretion of hGH about 10-fold compared with p0gh, whereas it does not function in 3T3 cells. The hGH gene under the control of the viral thymidine kinase promoter (pTKgh) used as a positive control for transfection is active in both cell lines.

Table 2. Functional analysis of mouse L/B/K-ALP promoter

The results are expressed as means ± S.E.M. for three independent transfections. The fold increase of the expression compared with the promoter-less plasmids is indicated in parentheses.

(*a*)

Promoter	Cells ...	CAT activity [total [^{14}C]acetyl chloramphenicol transformed (c.p.m.)] F9		3T3	
p106		2771 ± 146	(1)	4252 ± 111	(1)
pSV2CAT		427500 ± 40464	(158)	102696 ± 13228	(24)
ALPCAT		20344 ± 1363	(7)	8969 ± 1238	(2)
rALPCAT		7350 ± 907	(3)	4962 ± 15	(1)

(*b*)

Promoter	Cells ...	hGH (ng of immunoreactivity secreted/ml of cell culture medium) F9	3T3
p0gh		0.5 ± 0.1 (1)	0.4 ± 0.1 (1)
pTKgh		32.4 ± 3.8 (65)	52.1 ± 5.8 (148)
ALPgh		5.2 ± 0.3 (10)	0.8 ± 0.1 (2)

```
-1838   GAATTCTAGAGGGTCAGAGACCAACAACAGGGAAAGTGTAGCGAAGAAGGTGGCAGAAGG
-1778   AGATCAGCAGCCACTCCGATCCCTTGGAGAGTGCTTTCGATTCTCCATGAAGACACAATG
-1718   GACTCAGGCTAGCCATAAGTCAAGCCAGAATTGCTCATTCTCCATGTGTGGTGTTGGGGG
-1658   CAGAGGAGAGGGGCTCCGTGGTAAGAGACAGGACCCTTCAGCCTTCAGCGCCTGCTGCTT
-1598   CAGCTTCTCAGCTCTGCCAATGTTGGGAAGGGGGATCATTTATGATGCGGATGTCCCTCG
-1538   GCACACAGATACAATCTCCAGCCTTTTGTCATCTTGAGCCCTTTGGCGCTAGCTAGCAAG
-1478   TAATTGCCAAGTGCTGTGTTCTGAGCTCACTGCTGTCTTAACTCTCAGCTCCTTACAGAC
-1418   GAGGACTGCCGGGAAATGACCAAAGTTTCATTTATTCACCGCCCCAATGGCTTTTGCCAT
-1358   TATGACCTTGCTATGAAATACATAAATAAGGAGTAATTTACCCAATACTTGGTCTCACTT
-1298   TGTAAAAAGATAAACTTATTTCTCAAGGGAAATTTCCTAGCACCGCTGTAAATAAACAGC
-1238   ATTAATTATTAAAATACAAGAACAAATGAAAACAAACCCAGGCATTCCTGGGTTTTGGCA
-1178   TCAGGCTCCAGTTCCGGCCCTGAACTTACCGATGAGGTCACATGTTGTCTCTGGGCCTTG
-1118   CTTTTCCTCCTGTAAAATGGGCTCAATAGTAGAGCTCATATAGAAGTAGCCTGGACCTAA
-1058   GACGGAACTTCAGGGTAGAAGTGATCAATCCTAGGTGCCCAGGAAGATGGAGTTCAGGGA
- 998   AACCTGATCTTTTGCTTTCAAATCTCTGGCTGTTGGGGATTAGAGGTACAGATGGGTTTT
- 938   GTCAGCTTCAGTAGCGATGATAAGACCCTCCTCTTAGGAGGAAATGAGGAAGTATTCAAA
- 878   TGGTGGTGGCATTCAAACCTACCTCCAGGAGTTCACAGTAGGAAACGAATCAGAGACTTC
- 818   AAAACCAGACTGGCTTTCAGGGGAAGGGAGGTGGTGGCCCTTGGGGAACTGGATAAGGCC
- 758   CACTGGCACCCAGCGGTTGCCTTTCCTTGTTTCCTGTGCATGTTCCAAACTCGGTCTCTT
- 698   ATAACTCTCCCAATAACGTGAAGGAAGAAAAACCAGGGGATAGTGTCTGCGGGTTATAGA
- 638   GCCTTGGCCAGTCCCCCGAAGTCACCAGAGCAGCCTGTGGGCCTAGACAGTCTGGCTCCA
- 578   CTGCCTGGGAAGTGGTCTGCCCCGGGCAGGTCCTGGGGCTTCTGGCTGGGACAGACAGAA
- 518   TGTCCTGAGTCCCTGTTTCTTCAGATATTAGGGAGGGAGGGCCCTGTCCAAGGGGTCTCC
- 458   TTCTGCTTCCTACTGACTTAGTTAAGGGCAAAAGAGAAAGTGGATAGGAAAGGGGTAAGG
- 398   GTGTGAGGCTCAGAGGTGCACATGGTGACGGACAAGGATGTGTAGGCAGAAAGACGCACA
- 338   GAAAAAAGGAGCCAGGTGGGTTCACAGACGCGCAGGGTCAGGCACAGGAAGACAACCATC
- 278   GAGGGACAAAGTCTCCTACAGAGAAATCAAACCTAAAGACAAGCCAGCTGCACCCTGAAG
- 218   CCAGGATGAGCCGCAGGGAAAGAGAGAGGCAAGGCGGGTTACCCTGCCTGTTGCAGCCCT
- 158   ACGGGCCGGGTCGAGCCCGCTCCCGGCAGGGGGCGCCCTGGCAACGCAGAGGCACCCGCC
-  98   TGGGGCTCGGGGTGCGGGCCGGGGGCGGGGCGGCCGGGGGCGGGGAGGCCGGCGGGTGCT
-  38   CGGCCAGGCCGCCTTCATAAGCAGGCGGGGGAGGTGGCCGCCAGAGTACGCTCCCGCCAC
+  23   TGCGCTCCTTAGGGCTGCCGCTCGCGAGCCGGAACAGACCCTCCCCACGAGTGCCTGCAG
+  83   gtaaggaaagcatcgtgc ---------- INTRON 1 ----------
```

Fig. 4. Primary structure of the 5′-flanking region of mouse L/B/K-ALP gene

The putative transcriptional initiation site is numbered as +1, and is indicated by a dot. Upstream sequences are indicated by negative numbers. The 5′-flanking sequence as well as the first exon (underlined with a broken line) is shown in capital letters. Lower-case letters indicate part of the sequence of intron 1. A TATA-like sequence is boxed, and three copies of the consensus sequence for Sp1 binding are underlined. Horizontal arrows above the sequence indicate a 14 bp direct repeat and a 9 bp inverted repeat. Multiple copies of a purine-rich direct repeat are doubly underlined.

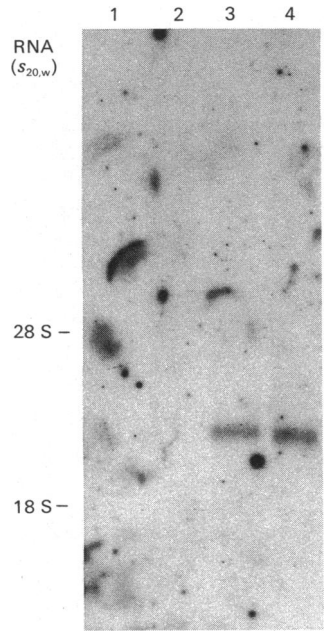

Fig. 5. RNA blotting analysis of mouse L/B/K-ALP mRNA

Cellular RNA from 3T3 fibroblasts and undifferentiated F9 teratocarcinoma cells were fractionated on a 1%-formaldehyde/agarose gel, transferred to a nylon membrane and hybridized to ^{32}P-labelled mouse placental cDNA. Lanes 1 and 2, 10 and 20 μg of 3T3 RNA respectively; lanes 3 and 4, 10 and 20 μg of F9 RNA respectively. The positions of ribosomal 28 S and 18 S RNA species are indicated.

With both types of construct, the promoter activity of the ALP gene in F9 cells is lower than that of SV40 promoter–enhancer and pTKgh (5 and 16% respectively).

DISCUSSION

The mouse L/B/K-ALP gene is at least 49 kb in size and consists of 12 exons. The main feature of this gene is the presence of a long first intron (at least 32 kb) and the clustering of all the other exons in the remaining portion. Several attempts to get overlapping clones within intron 1 using various genomic libraries and probes derived from the isolated gene were unsuccessful, probably because of the presence of repetitive sequences in this region. It is unlikely, however, that clones 81 and 51 (see Fig. 1) come from a gene different from, but closely related to, that represented by the remaining clones. In fact, the splicing junction between exons 1 and 2 is perfectly matched (see Table 1), and the results of DNA blotting analysis of mouse genomic DNA are consistent with our physical map of the ALP gene and with the absence of other related genes (see Figs. 1 and 2).

The similarity of the nucleotide sequence of the mouse placental ALP cDNA to that of its human and rat L/B/K-ALP counterparts in the coding region is 84% (Weiss *et al.*, 1986; Terao & Mintz, 1987) and 92% respectively (Misumi *et al.*, 1988). The structural organization of the mouse gene is also strikingly similar to the rat and human L/B/K-ALP genes (Toh *et al.*, 1989; Weiss *et al.*, 1988). One similarity among the three genes is the presence of a long first intron, which splits the 5′-untranslated region into exons 1 and 2. The organization of other exons is completely conserved among mouse, rat and human: both the length of the exons and the sites of interruption by introns are exactly the same. The length of the different introns, as well as their sequence in the characterized regions is, however, different. In particular, the sequences surrounding the exon–intron junctions are different from the ones found in the human L/B/K-ALP gene, but very similar to that of the rat. The fact that, in the mouse L/B/K-ALP gene, the protein coding region is interrupted by introns at positions analogous to those in the human genes encoding the three different ALP isoenzymes and in the rat L/B/K-ALP gene further strengthens the contention that this multigene family is the product of a series of duplications from a single ancestral gene (Weiss *et al.*, 1988).

Like the human L/B/K-ALP gene, the mouse counterpart has only one major capping site, which, within the limits of primer extension and S1 mapping analysis, matches the 5′ end of the mouse placental cDNA (Terao & Mintz, 1987). Preliminary experiments suggest that a single major transcription start site is also present in F9 teratocarcinoma cells. Unlike the human and mouse homologues, the rat L/B/K-type ALP gene has an additional promoter which allows the production of an extra mRNA from the same gene. The occurrence of alternatively spliced mRNA species of the ALP gene may, however, be a peculiarity of the rat, since there is no report of more than one molecular species of the L/B/K-ALP transcript in mouse and human. Moreover, attempts to evaluate the presence of a second promoter sequence by hybridization of our genomic clones 81, 51 and 21 with various oligonucleotides synthesized according to the published sequence of the rat L/B/K-type ALP exon 2 (Toh *et al.*, 1989) were negative. Primer extension analysis performed with the same oligonucleotides, as well as with other oligonucleotides in the coding region of mouse placental ALP cDNA, using polyadenylated RNA from mouse placenta, liver and F9 cells, again did not suggest the presence of an extra exon sequence similar to that of the rat gene.

The 5′-flanking sequences of the human and the mouse genes are very similar. When compared with the first 600 bp of the human L/B/K-ALP gene, more than 50% overall identity is observed, and this similarity is particularly high (78%) in the 200 bp upstream of the putative capping site (results not shown). The 5′-flanking region of the first exon of rat L/B/K-type ALP is even more similar to the mouse promoter, being 86% identical in the first 500 bp upstream of the putative capping site. The ALP promoter of the three genes is devoid of a TATA-box consensus sequence (Serfling *et al.*, 1985; Dynan & Tjian, 1985), although a similar sequence (TTCATAA in mouse and rat versus TTTATAA in human) is present. This feature, along with the lack of a CAAT-box element, is typical of many eukaryotic house-keeping genes (Serfling *et al.*, 1985; Bird, 1986). Three copies of Sp1-binding site, as compared with the four copies observed in the human and rat L/B/K-ALP promoter, are also found in the mouse ALP-gene promoter.

To demonstrate the functional activity of the 5′-flanking region of the mouse ALP gene as a promoter, a 1.9 kb fragment containing this region was tested for the expression of two different reporter genes in F9 teratocarcinoma cells and 3T3 fibroblasts. With both reporter genes, ALP promoter activity is observed in F9 cells, whereas the activity is lower or absent in 3T3 fibroblasts (compare the ALPCAT and ALPgh expression in 3T3 cells shown in Table 2). The two kinds of constructs using different reporter genes were produced to compensate for possible 'artifactual' differences in the level of expression of the ALP promoter in the two cell lines. The transient expression assay thus seems to be correlated with the ALP enzymic activity as well as specific mRNA steady-state levels in these two cell lines. This suggests that the 5′-flanking region contains general elements essential for transcription as well as determinants for cell specificity of transcription. In F9 cells, the mouse L/B/K-type ALP promoter is active in both orientations in respect to the transcriptional direction of the reporter gene, albeit at different levels. This phenomenon was also observed for the human L/B/K-type ALP gene, for which similar experiments were conducted in human osteosarcoma cells (Weiss *et al.*, 1988). The activity of the mouse L/B/K-ALP promoter in F9 cells is about 16% of that of the viral TK promoter and about 5% of the simian-virus-40 late antigen promoter–enhancer element. This would argue for the absence of an enhancer element in the DNA used for these experiments or for the presence of a suppressor element. Studies are needed to determine the regions of the mouse ALP promoter involved in the regulation of its expression both *in vitro* in different mouse cell lines and *in vivo* in the transgenic animal.

The isolated gene will be useful for the inactivation of the endogenous mouse L/B/K-ALP gene by homologous recombination in cultured embryonal stem cells (ES cells) according to procedures recently developed (Thomas & Capecchi, 1987; Schwartzberg *et al.*, 1989). This might allow the production of a transgenic mouse potentially useful as an animal model of the human disease known as hypophosphatasia.

This work was supported partly by grants from the Consiglio Nazionale delle Ricerche (CNR) and the Associazione Italiana per la Ricerca contro il Cancro (AIRC), by Mrs. Gigina Necchi Campiglio and Ms. Nedda Necchi.

REFERENCES

Atchison, M. L., Meyuhas, O. & Perry, R. (1989) Mol. Cell. Biol. **9**, 2067–2074
Benham, F. J., Fogh, J. & Harris, H. (1981) Int. J. Cancer **27**, 637–644
Berger, J., Gerattini, E., Hua, J. C. & Udenfriend, S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 695–698
Berk, A. J. & Sharp, P. A. (1977) Cell (Cambridge, Mass.) **12**, 721–732
Bernstine, E. G., Hooper, M. L., Grandchamp, S. & Ephrussi, B. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 3899–3903
Bird, A. P. (1986) Nature (London) **321**, 209–213
Birnstiel, M. L., Busslinger, M. & Strub, K. (1985) Cell (Cambridge, Mass.) **41**, 349–359
Bradford, M. (1976) Anal. Biochem. **72**, 248–254
Briggs, M. R., Kadonaga, J. T., Bell, S. P. & Tjian, R. (1986) Science **234**, 47–52
Chiquoine, A. D. (1954) Anat. Rec. **118**, 135–145
Dynan, W. S. & Tjian, R. (1985) Nature (London) **316**, 774–778
Feinberg, A. P. & Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13
Goldstein, D. J., Rogers, C. E. & Harris, H. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2857–2860
Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) Mol. Cell. Biol. **2**, 1044–1051
Graham, F. L. & Van Der Eb, A. J. (1973) Virology **52**, 456–467
Green, M. R. (1986) Annu. Rev. Genet. **20**, 671–708
Hamilton, T. A., Tin, A. W. & Sussman, H. H. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 323–327
Hass, P. E., Wada, H. G., German, M. M. & Sussman, H. H. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 1164–1168
Henikoff, S. (1984) Gene **28**, 351–359

Henthorn, P. S., Knoll, B. J., Raducha, M., Rothblum, K. N., Slaughter, C., Weiss, M. J., Lafferty, M. A., Fisher, T. & Harris, H. H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5597–5601
Henthorn, P. S., Raducha, M., Edwards, Y. H., Weiss, M. J., Slaughter, C., Lafferty, M. A. & Harris, H. H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1234–1238
Henthorn, P. S., Raduch, M., Kadesch, T., Weiss, M. J. & Harris, H. (1988) J. Biol. Chem. **263**, 12011–12019
Kadonaga, J. T., Jones, K. A. & Tjian, R. (1986) Trends Biochem. Sci. **11**, 20–23
Kam, W., Clauser, E., Kim, Y. S., Kan, Y. W. & Rutter, W. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8715–8719
Knoll, B. J., Rothblum, K. N. & Longley, M. (1988) J. Biol. Chem. **263**, 12020–12027
Lalley, P. A. & McKusick, V. A. (1985) Cytogenet. Cell Genet. **40**, 536–566
Lalley, P. A., Francke, U. & Minna, J. D. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 2382–2386
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Marth, J. D., Disteche, C., Pravtcheva, D., Ruddle, F. H., Krebs, E. G. & Perlmutter, R. M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7400–7404
Maxam, A. M. & Gilbert, W. (1980) Methods Enzymol. **65**, 499–560
McComb, R. B., Bowers, G. N., Jr. & Posen, S. (1979) Alkaline Phosphatase, Plenum Publishing Corp., New York
McKenna, M. J., Hamilton, T. A. & Sussman, H. H. (1979) Biochem. J. **181**, 67–73
Millan, J. L. (1986) J. Biol. Chem. **261**, 3112–3115
Mintz, B. & Russel, E. S. (1957) J. Exp. Zool. **134**, 207–238
Misumi, Y., Tashiro, K., Hattori, M., Sakaki, Y. & Ikehara, Y. (1988) Biochem. J. **249**, 661–668
Mount, S. M. (1982) Nucleic Acids Res. **10**, 459–472
Nadeau, J. H. & Taylor, B. A. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 814–818
Ovitt, C. E., Strauss, A. W., Alpers, D. H., Chou, J. Y. & Boime, I. (1986) Proc Natl. Acad. Sci. U.S.A. **83**, 3781–3785
Sanger, F., Nicklen, S. & Coulsen, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
Schwartzberg, P. L., Goff, S. P. & Robertson, E. J. (1989) Science **246**, 799–803
Serfling, E., Jasin, M. & Schaffner, W. (1985) Trends Genet. **1**, 224–230
Sergeant, L. E. & Stinson, R. A. (1979) Nature (London) **281**, 152–154
Swallow, D. M., Povey, S., Parkar, M., Andrews, P. W., Harris, H., Pym, B. & Goodfellow, P. (1986) Ann. Hum. Genet. **50**, 229–235
Terao, M. & Mintz, B. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7051–7055
Terao, M., Pravtcheva, D., Ruddle, F. H. & Mintz, B. (1988) Somat. Cell Mol. Genet. **14**, 211–215
Thomas, K. R. & Capecchi, M. R. (1987) Cell (Cambridge, Mass.) **51**, 503–512
Toh, Y., Yamamoto, M., Endo, H., Misumi, Y. & Ikehara, Y. (1989) Eur. J. Biochem. **182**, 231–237
Weiss, M. J., Henthorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M. & Harris, H. H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7182–7186
Weiss, M. J., Ray, K., Henthorn, P. S., Lamb, B., Kadesch, T. & Harris, H. (1988) J. Biol. Chem. **263**, 12002–12010
Wilcox, F. H. (1983) Biochem. Genet. **21**, 641–652
Wood, W. I., Gitschier, J., Lasky, L. A., & Levin, R. M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1585–1588

Received 1 December 1989; accepted 8 January 1990