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Genomic Organization of the OS-9 Gene Amplified in Human Sarcomas[1]

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The OS-9 gene is frequently amplified in human sarcomas. We isolated and characterized an OS-9 genomic DNA from a human BAC library. Sequencing of the genomic DNA showed that the gene spanned approximately 30.4 kbp and had 15 exons. The 1,010 bp sequence of the 5′ upstream region was also determined. The potential binding-sequence motifs TATA and CCAAT for general transcription factors were found in the 5′ upstream region. Primer extension analysis revealed two putative transcription start sites. The significance of the 5′ upstream sequence in the ubiquitous expression of the OS-9 gene in various tissues and culture cells is discussed.

Key words: human sarcomas, OS-9 gene, gene structure, 5′ upstream region, transcription start sites.

Amplification of genes frequently occurs in human tumors and leads to their overexpression, resulting in the induction of abnormal cell growth. The q13-15 region of human chromosome 12 is frequently amplified in human sarcomas, and several genes amplified in this region are *MDM2* coding for a regulator of the tumor suppressor protein p53 (*1*), *GLI* for a zinc finger protein (*2*), *GADD153/CHOP* for a member of the C/EBP family of transcription factors (*3*), *SAS* for a member of a transmembrane 4 superfamily (*4, 5*), *CDK4* for cyclin-dependent kinase 4 (*6*), and *A2MR* for a α-2 macroglobulin receptor (*7*). Two novel genes, *OS-4* and *OS-9*, have been isolated by chromosome microdissection and hybrid selection (*8*). The OS-9 gene is located near *SAS* and *CDK4* in a gene-dense region (*9, 10*). While isolating protein tyrosine phosphatase (PTP) cDNAs from an HL-60 cDNA library by hybridization with a PTP 1B cDNA probe, we found a novel cDNA (Kimura, Y. *et al.*, unpublished data), which has been identified as a reported recently OS-9 cDNA (*11*). It encodes a polypeptide of 667 amino acid residues. Features of the deduced amino acid sequence are a potential nuclear targeting sequence and an aspartic acid- and glutamic acid-rich stretch conserved in nucleolin, the major nucleolar protein of growing cells. The OS-9 gene is ubiquitously expressed in normal tissues (*11*) and various types of tumor cell lines, but it is expressed differentially during macrophage differentiation of human myeloid leukemia HL-60 cells (Kimura, Y. *et al.*, unpublished data). For full understanding of its expression, elucidation of its genomic structure is essential. In the present study, we isolated an OS-9 genomic DNA and characterized the structural organization and 5′ upstream region of the OS-9 gene.

MATERIALS AND METHODS

Chemicals—[γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from the Institute of Isotopes of the Hungarian Academy of Science. Hybond N nylon membranes were from Amersham Life Science. A digoxigenin (DIG) DNA labeling and detection kit was from Boehringer Mannheim. OligotexTM-dT30<Super> was from Daiichi Pure Chemicals. Oligonucleotides were synthesized by Nippon Bio Service.

Preparations of RNA and DNA—Total RNAs of the human promyelocytic leukemia cell line HL-60 and the human osteosarcoma cell line SJSA-1/OsA-CL (obtained from ATCC) grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (General Scientific Laboratories) and kanamycin sulfate (50 μg/ml) at 37°C under 5% CO_2 in air were isolated by the acid guanidium thiocyanate method (*12*). Nuclear DNAs were isolated as described previously (*13*).

Nucleotide Sequencing—The plasmid containing an insert was sequenced with an Li-COR dna sequencer model 4000L, using a cycle sequencing kit (Epicenter Technology). For analysis of PCR products, at least three plasmids were sequenced. The complete sequence was determined from the consensus sequences with GeneWorks software (Intelligenetics) and homology was searched using the BLAST and FASTA programs.

Cloning of an OS-9 cDNA—Approximately 1×10^6 plaques of an HL-60 cDNA library in λgt10 (*14*) were screened by hybridization on nitrocellulose filters at 50°C using ^{32}P-labeled PTP1B cDNA as a probe with a low stringent wash. A cDNA clone was obtained and named HOS-9. Its sequence of 2,690 bp was determined and found

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to be identical with that of a reported OS-9 cDNA (*11*), except for its 5′ 16 bp sequence.

OS-9 Gene Screening—A human-bacterial artificial chromosome (BAC) library consisting of 96,000 clones with an average DNA insert of 110 kbp was screened by two-step PCR using an OS-9 cDNA 3′-untranslated region-specific primer pair (nt 2260-2280 5′-TTG CTC TCC TGA ACT CTC ACT-3′ and nt 2617-2597 5′-ATT GTG CTG GAG GTG ATA GGC-3′) as described previously (*15*). Two clones, 257B2 and 800A3, containing an overlapping genomic DNA insert were obtained. To identify the OS-9 genomic region of each clone, Southern hybridization was performed. These two BAC plasmids were prepared by the alkali lysis method (*16*), digested with several restriction enzymes, electrophoresed in 0.7% agarose gel, and transferred to nylon membranes. The membranes were hybridized with various DIG-labeled fragments of OS-9 cDNA for 15-20 h at 65°C. A restriction map of the OS-9 genomic region was constructed. The OS-9 genomic restriction fragments were subcloned into a multiple cloning site of pGEM7Zf or pBluescriptSK(−) and sequenced.

Rapid Amplification of the cDNA 5′ End (5′ RACE)—5′ RACE was carried out essentially as described with minor modifications (*17*). Poly(A)$^+$ RNAs were purified from total RNAs of HL-60 cells and SJSA-1 cells using oligotex-dT30<Super>. First strand cDNA was synthesized from 2 μg of poly(A)$^+$ RNA using a 1st antisense primer (nt 427-409 5′-TGG ATG TGG CGT CCA TAA C-3′) and 200 units of Moloney murine leukemia virus reverse transcriptase (Superscript II, Gibco BRL) in a total volume of 20 μl. Remaining primers were removed using a Sephadex G-50 spun column (Pharmacia), and the cDNA was polyguanylated at its 3′ terminus using 15 units of terminal deoxynucleotidyl transferase (Gibco BRL) by incubation with 0.1 mM dGTP at 37°C for 15 min. In the first round polymerase chain reaction (PCR), a tenth portion of the polyguanylated template was amplified using a C primer (5′-GGC CCG ACG TCG CAT GAA TTC GCC CCC CCC CCC C-3′), a 2nd antisense primer (nt 230-211 5′-GAC AAT CAC CAC GTC CGA AG-3′) and an *Apa*I primer (5′-GGC CCG ACG TCG CAT G-3′) for 1 cycle consisting of denaturing for 2 min at 95°C, annealing for 2 min at 50°C and extension for 40 min at 72°C and 35 cycles consisting of denaturing for 40 s at 94°C, annealing for 2 min at 55°C and extension for 3 min at 72°C. The PCR products were directly subcloned into a pCR II vector (TA cloning kit, Invitrogen) and sequenced.

Primer Extension—The 5′ termini of OS-9 mRNA were analyzed by primer extension as described (*16*). The reaction mixture (6 μl) consisted of 5.2 μg of HL-60 poly(A)$^+$RNA, 3 pmol of ^{32}P-labeled 5′ antisense primer (nt 48-32, 5′-CCGCCATCTTTCGTTCC-3′), 60 units of Superscript II reverse transcriptase, 6 units of placental RNase inhibitor (Pharmacia), 6 nmol of the four dNTPs and was incubated at 42°C for 2 h. The reaction was stopped by the addition of 4 μl of loading buffer. The lengths of the reaction products were determined by 8% polyacrylamide sequencing gel electophoresis with reference to the sequence of an OS-9 genomic 5′ flanking region of a p1.8EB clone analyzed with the same labeled primer using a sequenase sequencing kit (United State Biochem.).

Southern Blotting—The 3′ fragment (2,289 bp) and 5′ fragment (366 bp) obtained from an *Eco*RI digest of OS-9 cDNA were labeled with [α-^{32}P]dCTP by the random primer DNA labeling method (*18*) and used as probes in Southern hybridization. For Southern hybridization, high molecular weight genomic DNAs were purified and digested with *Eco*RI. The digested DNAs (5 μg/lane) were electrophoresed in 0.7% agarose gel. Denatured DNAs were transferred from the gels to Hybond-N nylon membranes and UV-cross linked. Hybridization was performed at 42°C in 50% formamide, 6×SSPE, 0.1% SDS, 0.1 mg/ml of sonicated heat-denatured salmon sperm DNA, 5×Denhardt's solution, 5% dextran sulfate, and ^{32}P-labeled cDNA probe for 24-48 h. The filters were washed in 2×SSC/0.1% SDS at room temperature and in 0.1×SSC/0.1% SDS at 65°C, then exposed to X-ray films at −80°C.

RESULTS

Isolation and Characterization of OS-9 Genomic DNA from a BAC Library—We obtained two OS-9 genomic clones, 257B2 and 800A3, from a BAC library. A physical map of the OS-9 genomic region on these genomic DNAs was constructed by Southern blot hybridization with various portions of the OS-9 cDNA using the restriction enzymes *Eco*RI, *Bam*HI, *Sma*I, and *Hin*dIII (Fig. 1A), then the fragments were subcloned into plasmids for sequencing. The 257B2 clone contained the whole sequence of the OS-9 cDNA, whereas the 800A3 clone lacked the 5′ region. We determined the sequences of all exons and the exon-intron junctions (Table I). All intron sequences except that of intron 5 were read in one direction, and the sequence of one strand was overlapped with that of another (Fig. 1A). Intron 5 was estimated to be about 22.4 kbp long by agarose gel electrophoresis of restriction fragments. However, the presence of closely located two *Bam*HI sites could not be excluded. The gene consisted of 15 exons and 14 introns (Fig. 1B), and spanned approximately 30.4 kbp. All the exon-intron junctions followed the GT-AG rule, except for GC-AG in intron 11. The initiation codon is located on exon 1. The potential nuclear targeting signal is encoded by exon 10. The ED-rich domain is encoded by exon 11. Exon 15 is the largest exon and contains parts of the coding and 3′-untranslated regions.

Structure of the 5′ Flanking Region of OS-9 Genomic DNA—Figure 2 shows the sequence of the 5′ upstream region of OS-9 genomic DNA. To define the 5′ termini of OS-9 mRNA, 5′ RACE cDNAs were obtained by reverse transcription and PCR using HL-60 poly(A)$^+$ RNA as a template with two antisense primers (nt 426-407 and nt 230-211) and sequenced. The determined sequence of the 5′ portion of a cDNA is shown as exon 1 (nt 1-203) in the sequence of the genomic DNA (Fig. 2). On the other hand, in primer extension analysis, an antisense primer (nt 48-32) designed to be adjacent to the 5′ end of exon 1 was extended to the 5′ end of mRNA using HL-60 poly(A)$^+$ RNA as a template, and the length and 3′ position of the extension product were determined and mapped with reference to the sequence of genomic DNA determined with the same primer. Figure 3 shows that there were two major products. The 3′ end of the shorter product was mapped at nt 1, coincident with the 5′ end of the RACE cDNA, and that of the longer product was mapped at nt −18 (Fig. 2). These results together indicate that one of the transcription start sites is nt 1. Two other minor products were mapped at nt −8 and −27. The sequence of the 5′ immediately

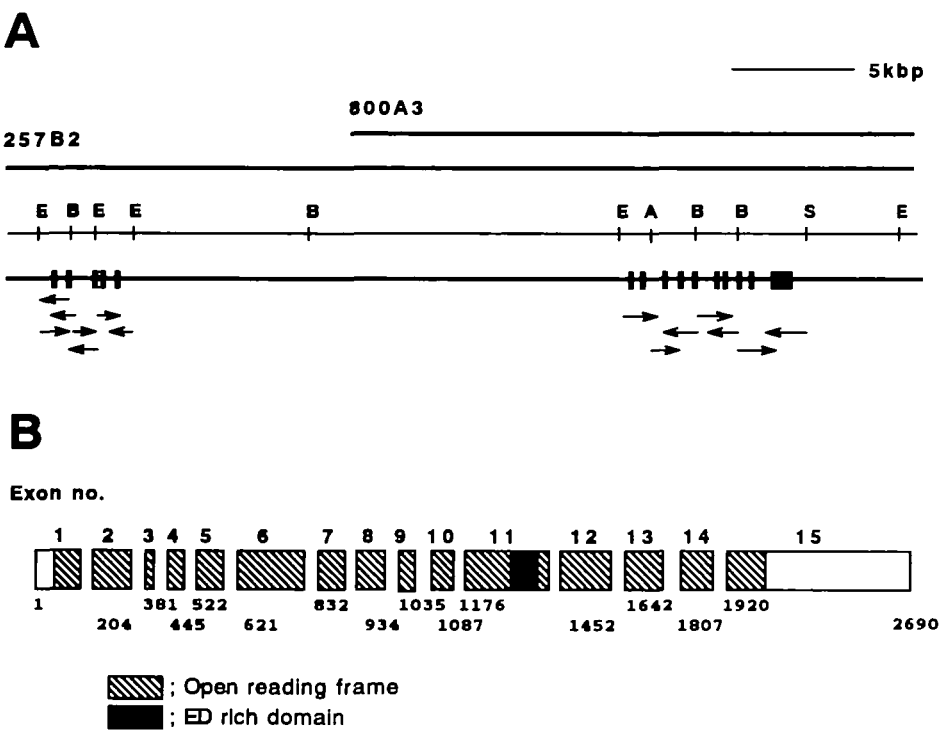

Fig. 1. **Structural organization of the OS-9 gene.** (A) Restriction map of the OS-9 gene. The genomic clones 257B2 and 800A3 were digested with various restriction enzymes, then analyzed by Southern blotting with various portions of OS-9 cDNA as a probe. The restriction fragments were subcloned and sequenced. The solid line and black boxes show introns and exons, respectively. Arrows that run parallel to the solid line indicate the direction and extent of sequencing. Only the restrition enzymes used for subcloning are shown on the map. E, *Eco*RI; B, *Bam*HI; A, *Apa*I; S, *Sma*I. (B) Relationship of exons and the coding region of the OS-9 cDNA. Each box represents one exon. Exon numbers are shown above the boxes. Numbers below the boxes show the position of the first nucleotide of each exon. Numbers below exon 15 show the position of the first and last nucleotide numbers of the exon. Position of the ED-rich region in OS-9 protein is also shown.

TABLE I. **Exon-intron junction sequences of the OS-9 gene.** Intron size was determined by sequencing, which was read in one direction and included ambiguous nucleotides. The nucleotides indicated in capitals are those of exon sequences and the lower case nucleotides are those of intron sequences.

Exon No.	Size (bp)	Intron donor	Size (bp)	Acceptor
1	203	GG CAG gtgagagg	424	tttcccag AGC CA
2	177	TG AAG gtgaaagg	850	attgtcag ACA AA
3	64	G GAA G gtaactca	118	ccttgcag AT TCA
4	77	CC AAG gtggcaag	236	ttctccag GCG TC
5	99	TT CGG gtgagtct	22,400[a]	cctggcag TTC CT
6	211	A GCC G gtgagtaa	121	ctcagtag AC TCA
7	102	G GCA G gtaggacc	220	taccctag GT GCG
8	101	CT GAG gtgagacc	181	ctcgtcag GAG CA
9	52	C AAT G gtgagtga	1,146	catctcag AT TTT
10	89	AA AAG gtataggc	186	ccacccag GGG AA
11	276	AG GAG gcaagccc	571	tattacag ACA GA
12	190	T ACA G gtgagagc	916	cctctaag AG GAG
13	165	T GCA G gtgggccc	142	ctttccag GG AAA
14	113	TC TTG gtaagagg	216	cccaccag GTG CC
15	771			

[a]Approximate size was estimated from the mobilities of *Bam*HI fragments with reference to λ*Hin*dIII fragments separated by agarose gel electrophoresis.

upstream region revealed binding sites for general transcription factors such as an atypical TATA box for TF IID and a CCAAT box for C/EBP. These features are compatible with the ubiquitous expression of the OS-9 gene. A sequence further upstream contained several binding sites for SP-1, AP-2, GATA, and NF-κB, but it is unknown whether these sites are involved in regulation of expression of the OS-9 gene.

Southern Blotting—*Eco*RI digests of DNAs obtained from HL-60 cells, human placenta and SJSA-1 cells bearing an hsr of 12q13-15 were analyzed by Southern blotting using a 5′ cDNA fragment (366 bp) as a probe. Only a single band of approximately 2.4 kbp was detected, and its signal intensity was 3-4-fold stronger in SJSA-1 cell DNA than in HL-60 cell and placental DNAs (data not shown). FISH using a 3′ cDNA fragment of 2,289 bp as a probe confirmed that the gene is localized on the band q13 of chromosome 12. These results suggest that the OS-9 gene is present as a single gene per haploid.

DISCUSSION

This work indicated that the OS-9 gene covered approximately 30.5 kbp and contained 15 exons. *CDK4* and *SAS* have been mapped to 12q13 by FISH (*6*) and physically placed in close proximity to *OS-9* (*9, 10*). As predicted from these findings, FISH revealed that *OS-9* mapped to the band q13 (data not shown). No other positive signal on the chromosome was detected. The 12q13-15 region is frequently amplified in human sarcomas and malignant gliomas (*19, 20*). Several genes in this region are amplified at different frequencies in tumor samples. Either *MDM2* or *CDK4* or both these genes are the most frequently amplified (*19, 21*). MDM2 and CDK4 are both involved in cell cycle regulation by interacting with the tumor suppressor p53, the retinoblastoma repressor protein pRB and S-phase inducing transcription factors E2F1/DP1(*1, 6, 22-25*). It is considered that *MDM2* and *CDK4* are the targets responsible for 12q13-15 amplification.

Physical mapping of 12q13-15 hsr region has shown that *MDM2* and *CDK4* are located several mbp apart (*26*). *SAS* and *OS-9* are located within 10 and 40 kbp, respectively, of *CDK4* (*9, 10*). *CDK4* and *SAS* are amplified at similar, high frequencies, which are slightly higher than that of *OS-9* (*19*). Therefore, *OS-9* is probably co-amplified with *CDK4*, because of the physical proximity of these genes.

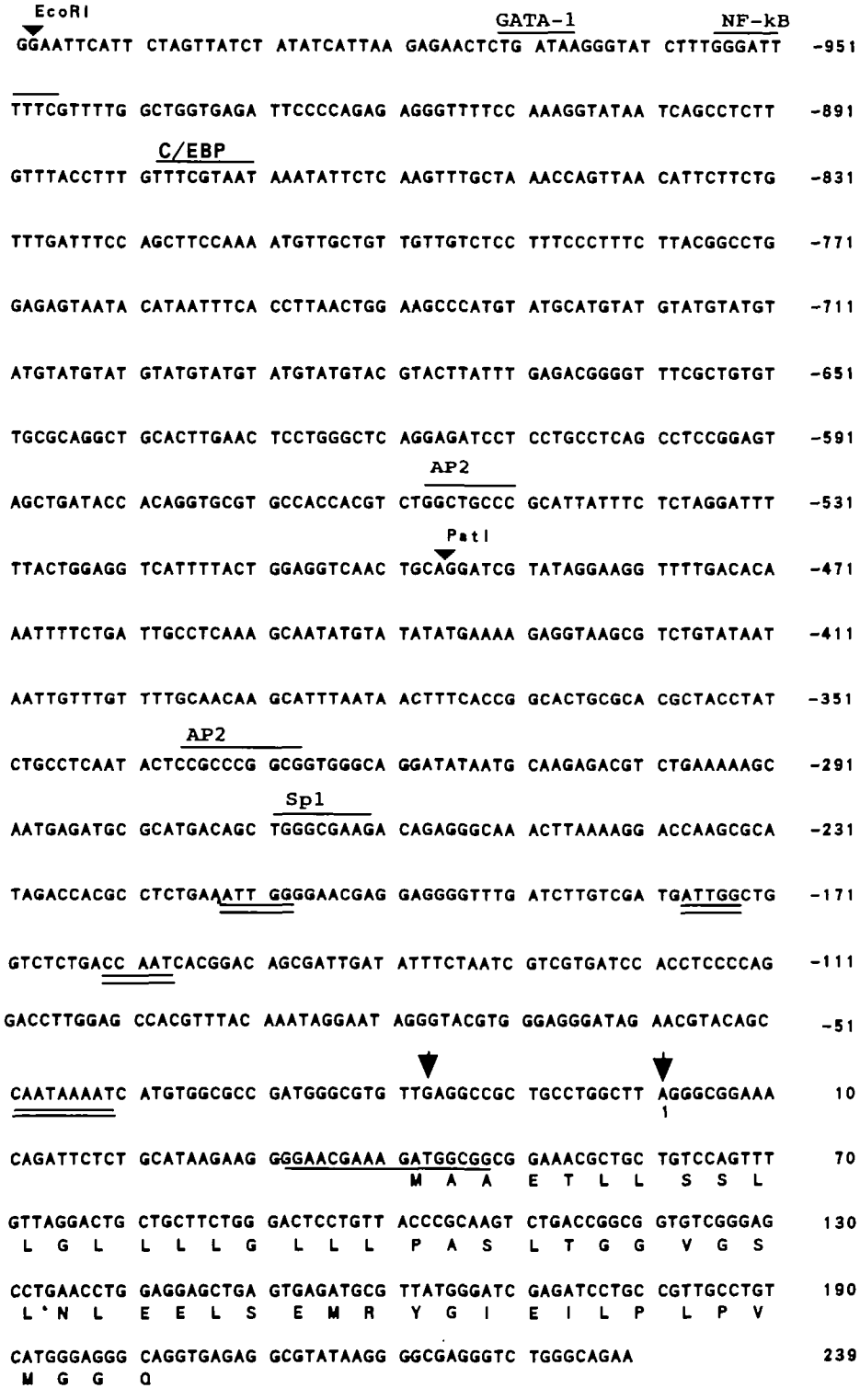

Fig. 2. **Sequence of the 5′ upstream region and 5′ portion of the OS-9 gene.** Nucleotide positions are numbered relative to the first nucleotide of exon 1. The 5′ end of the 5′ RACE cDNA is taken as +1. Arrows above the sequence indicate the positions of the 3′ ends of the major primer extension products mapped based on their lengths. The underline indicates the position of the antisense primer used for primer extension. Double underlines at nt −50, −162, and nt −178 and −213 indicate the putative TATA box, the CCAAT box, and the ATTGG boxes, respectively. Putative transcription factor binding sites are overlined. Restriction sites used for subcloning are shown. Nucleotides from 1 to 203 are exon 1, and their encoded amino acids are shown in the bottom row.

Amplification of the OS-9 gene in human osteosarcoma SJSA-1 cells bearing an hsr of 12q13-15 was exemplified.

OS-9 cDNA was isolated as a cDNA clone crosshybridized with a PTP1B cDNA probe. However, the sequence of the cDNA was not related to that of PTP1B cDNA, except that its 95 bp sequence had 50% homology with a portion of the cDNA probe. The biological function of OS-9 is still unknown. Its gene is expressed in all normal tissues and all culture cells examined (*11*, Kimura, Y. *et al.*, unpublished data). To understand the expression of the OS-9 gene in various tissues and various types of culture cells, elucidation of the sequence of the 5′ region of OS-9 gene was essential. The determined upstream sequence indicated that the canonical CCAAT sequence and an atypical TATA sequence (CAATAAT) are located at nt −162 and −50, respectively. In addition to the OS-9 gene, several cell-

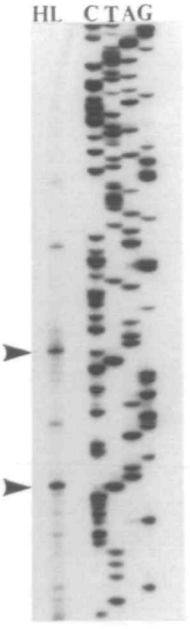

Fig. 3. **Primer extension.** The 5′ termini of OS-9 mRNA were analyzed by primer extension as described under "MATERIALS AND METHODS." A 5′ ^{32}P-labeled antisense primer (17 mer) for OS-9 mRNA was extended using HL-60 poly(A)$^+$ RNA as a template. Arrows show positions of the major extension products (lane HL). Lanes C, T, A, and G show sequence ladders of the OS-9 genomic 5′ flanking region made with the same ^{32}P-labeled primer.

cycle-regulated or housekeeping genes also lack traditional TATA or CCAAT sequences (*27, 28*). The absence of an authentic TATA sequence may account for the findings of the two major transcription start sites separated by 18 nucleotides. Another interesting feature of the 5′ upstream sequence is the presence of two reverse complements of CCAAT (ATTGG) located at nt −178 and −213 and the Sp1 binding site at nt −270. An array of CCAAT boxes and Sp1 sites is also common in the 5′ upstream sequences of a number of cell-cycle-regulated genes (*28*). These sequence features in the 5′ region of OS-9 gene are consistent with the ubiquitous expression of the gene. The genomic organization including the 5′ regulatory sequence of the OS-9 gene will aid in understanding OS-9 expression and the physiological role of the OS-9 protein in cells.

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