Biochimica et Biophysica Acta 825 (1985) 140–147
Elsevier

BBA 91458

Comparative analysis of the human and feline c-*sis* proto-oncogenes. Identification of 5′ human c-*sis* coding sequences that are not homologous to the transforming gene of simian sarcoma virus

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(Received January 3rd, 1985)

Key words: Proto-oncogene; Restriction enzyme analysis; (Feline, Human)

Feline and human genetic sequences, homologous to the v-*sis* gene of simian sarcoma virus, have been isolated from cosmid gene libraries and characterized by restriction endonuclease analysis. Comparison of the two loci revealed their related structural organization. In both loci, similar unique genetic sequences were found upstream of the v-*sis* homologous region and these hybridized to a 4.2 kbp c-*sis* transcript in human lung tumor cells. These data establish and map as yet unidentified coding sequences at the 5′ part of the c-*sis* proto-oncogene of both species.

Introduction

It is well established that viral oncogenes of acutely transforming RNA tumor viruses have arisen by recombination between retroviral genomes and genetic sequences of cellular origin [1–3]. These cellular sequences, which are highly conserved during evolution, are known as proto-oncogenes. In their normal form, they are thought to have an important function during growth and differentiation [4–7]. It has also been suggested that proto-oncogenes are involved in tumorigenesis [8–11]. In that case, however, genetic changes such as point mutations [12], translocations [13] or insertions [14] seem to be required. Some viral oncogenes code for proteins that have been identified and characterized as a protein kinase capable of phosphorylating tyrosine residues [15,16], a growth factor [17,18] or a growth factor receptor [19].

The proto-oncogene under investigation in the present study is c-*sis*. Genetic sequences from this locus have been captured in the genome of Parodi-Irgens feline sarcoma virus (PI-FeSV), isolated from a fibrosarcoma of a cat infected with feline leukemia virus (FeLV) [20], and simian sarcoma virus (SSV), isolated from a fibrosarcoma of a woolly monkey [21]. Their transforming proteins have been identified as a 76 kDa fusion protein containing *gag* and *sis* genetic sequences [22] and 28 kDa protein [23–25], designated $p28^{sis}$, respectively. Recently, comparison of amino acid sequences of human PDGF-2 with predicted sequences of $p28^{sis}$ has shown that these proteins share a virtually identical region [17,18,25]. In the light of the fact that in certain human tumors transcripts have been found that are related to v-*sis* [26,27] and that a number of human tumors

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Abbreviations: RFLP, restriction fragment length polymorphism; ALL, acute lymphocytic leukemia; SSV, simian sarcoma virus; PI-FeSV, Parodi-Irgens feline sarcoma virus; FeLV, feline leukemia virus.

produce PDGF-like proteins [28,29], it is of interest to investigate the potential involvement of the *sis* locus in tumorigenesis. As a first approach, studies were focussed upon the isolation and characterization of this locus and its flanking cellular sequences. The size of the viral oncogene in SSV is about 1 kbp [23,24,30] and homologous transcripts found in human tumor cell lines are about 4.2 kbp and 2.7 kbp in size [27]. This could indicate that the human c-*sis* locus contains more genetic sequences than those homologous to v-*sis*. To resolve this, we have molecularly cloned the human and feline c-*sis* proto-oncogenes for comparative analysis and have performed Northern blot analysis of c-*sis* transcripts in human tumor cells.

Materials and Methods

Cosmid, phages, plasmids and E. coli strains

pC60, a recombinant of pBR322 containing the entire SSV provirus [31], was a gift from R.C. Gallo. FAO1 and FAO2 represent subclones of pC60 in phage vector m13mp8. They contain the 0.5 kbp *Sac*I/*Sma*I or the 0.5 kbp *Sma*I/*Xba*I restriction fragments of pC60, respectively. Both fragments together represent almost the complete v-*sis* oncogene. Restriction endonuclease *Sac*I cleaves the oncogene at nucleotide position 22 and *Xba*I just before the last nucleotide of *v-sis*. *Bam*HI linkers were used in preparation of the subclones.

pAO70, pAO73, pAO79 and pAO121 are subclones of the human c-*sis* locus (see also Fig. 3). pAO70 consists of a 5.0 kbp *Hin*dIII DNA fragment subcloned in pSVBR94. An internal 3.8 kbp *Kpn*I/*Kpn*I fragment of this insert was used as a molecular probe. pAO73 and pAO79 are recombinant clones of pBR322 containing respectively an 1.7 kbp *Bam*HI and an 1.3 kbp *Bam*HI/*Hin*dIII restriction fragment. A 2.0 kbp *Eco*RI/*Hin*dIII restriction fragment was subcloned in pUC18 resulting in pAO121.

E. coli strain HB101 (hsdR$^-$, recA$^-$) was used as a host for pC60, pAO70, pAO73, pAO79 and pAO121. *E. coli* strain 1046 (supE, supF, hsdS$^-$, recA$^-$) was used for preparing the pJB8 [32] cosmid libraries. The recombinant m13mp8 bacteriophages FAO1 and FAO2 were propagated in *E. coli* strain JM101 [33].

Preparation of DNA probes and hybridization

Preparation of DNA probes and their nick translation [34] was carried out as described before [35]. Primer extension reactions were according to Messing and co-workers [33]. The specific activity of the probes used in the hybridization studies was $(2–5) \cdot 10^8$ cpm/μg. Agarose gel electrophoresis, Southern blot [36] and hybridization analysis was performed as described before [35].

Molecular cloning of c-sis sequences

The human and feline v-*sis* homologous genetic sequences were isolated from previously constructed cosmid gene libraries [35,37]. High-molecular-weight DNA used for the construction of the cosmid gene libraries was prepared from blood cells of a patient suffering from a common type acute lymphocytic leukemia (ALL; patient No. 1283) and from lung tissue of a domestic cat. Southern blot analysis of DNA isolated from the leukemia patient indicated that the c-*sis* proto-oncogene of this patient was similar in structure to that found in normal human liver DNA (data not shown) and, thus, the previously constructed library was suitable for the isolation of the c-*sis* proto-oncogene.

RNA isolation and Northern blotting

Total cellular RNA was isolated according to the guanidine-thiocyanate procedure described by Feramisco et al. [38]. Upon poly(A) selection by oligo(dT)-cellulose chromatography, RNA was dissolved in sodium phosphate buffer (25 mM, pH 6.5), which contained DMSO (50%) and glyoxal (0.5 M), and heated to 50°C for 1 h. RNA was size fractionated by agarose gel electrophoresis (1.4% agarose gel) and transferred to nitrocellulose for hybridization analysis as described before [39].

Results

Analysis of genetic sequences homologous to v-sis in human and feline genomic DNA

For analysis of the size and distribution of genetic sequences in human and feline genomic DNA homologous to v-*sis* two v-*sis*-specific probes, FAO1 and FAO2 were used. These probes contained the 5′ and 3′ half of the viral oncogene as described under Materials and Methods. Hu-

man DNA, isolated from the ALL patient (No. 1283) and digested with restriction endonuclease *Hin*dIII (Fig. 1, lane 1), revealed a hybridizing fragment of about 23 kbp. A similar analysis of DNA isolated from blood cells of a normal individual (No. 040883) revealed three hybridizing fragments of about 23 kbp, 17 kbp and 5.7 kbp (Fig. 1, lane 2), indicating the presence of a *Hin*dIII restriction fragment length polymorphism (RFLP). Among 10 human DNA preparations tested in this way, 6 had the same *Hin*dIII RFLP in one allele, but none of the DNAs showed the *Hin*dIII RFLP in both alleles (data not shown). Southern blot analysis of DNA of ALL patient No. 1283 with restriction endonuclease *Eco*RI revealed a single hybridizing fragment of about 25 kbp, and with restriction endonuclease *Xba*I, three v-*sis* homologous DNA fragments of about 25 kbp, 8.4 kbp and 3.6 kbp were identified (data not shown).

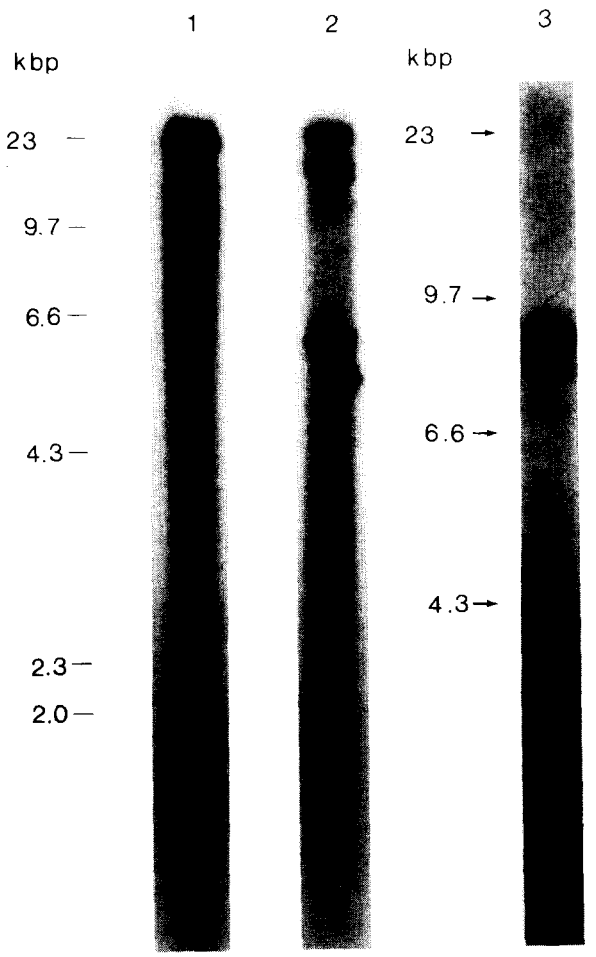

Fig. 1. Identification of genetic sequences in human and feline cellular DNA homologous to v-*sis*. High-molecular-weight DNA was prepared from blood cells of a patient suffering from a common type acute lymphocytic leukemia (patient No. 1283) (lane 1) and blood cells of a normal individual (lane 2) or from lung tissue of a domestic cat (cat No. 031182) (lane 3), digested with restriction endonuclease *Hin*dIII (lane 1 and 2) or *Eco*RI (lane 3) and size-fractionated by agarose gel electrophoresis through an 0.7% agarose gel. Upon transfer of the DNA to nitrocellulose, hybridization analysis was performed with ^{32}P-labeled probes (FAO1 and FAO2) representing almost the complete v-*sis* oncogene. Molecular weight markers included are *Hin*dIII-digested λ DNA fragments.

Southern blot analysis of high-molecular-weight feline DNA (cat No. 031182) with FAO1 and FAO2 is shown in lane 3 of Fig. 1. Two *Eco*RI hybridizing fragments could be identified. One was about 8 kbp in size and the other 4.3 kbp. Southern blot analysis with restriction endonuclease *Bam*HI revealed a 13 kbp, a 4.8 kbp and an 1.3 kbp v-*sis* homologous DNA fragment (data not shown). Similarly, DNA fragments of about 25 kbp, 5 kbp and 2.6 kbp were found using restriction endonuclease *Hin*dIII (data not shown).

These results indicate that in both species the v-*sis* homologous genetic sequences are distributed discontinuously over a relatively large region. Similar observations have previously been reported for the human locus [40–42]. Furthermore, in view of the fact that the viral oncogene of SSV seems to represent only a minor portion of the human cellular locus, the size of the complete proto-oncogene could be much greater.

Molecular cloning of human and feline c-sis genetic sequences

Based upon the results described above, we have used a previously described human and feline cosmid gene library to isolate from both species a large contiguous DNA region that contained the c-*sis* proto-oncogenes. Screening of the human cosmid gene library (300 000 colonies) with the v-*sis*-specific probes FAO1 and FAO2 resulted in one c-*sis* containing cosmid clone (ALLW-1283-Cl21) as is shown in Fig. 2A, lane 1 and Fig. 2B, lane 1. ALLW-1283-Cl21 contained all human v-*sis* homologous sequences as detected in genomic blot analysis (hybridization data not shown). A restriction map of this clone was obtained on the basis of

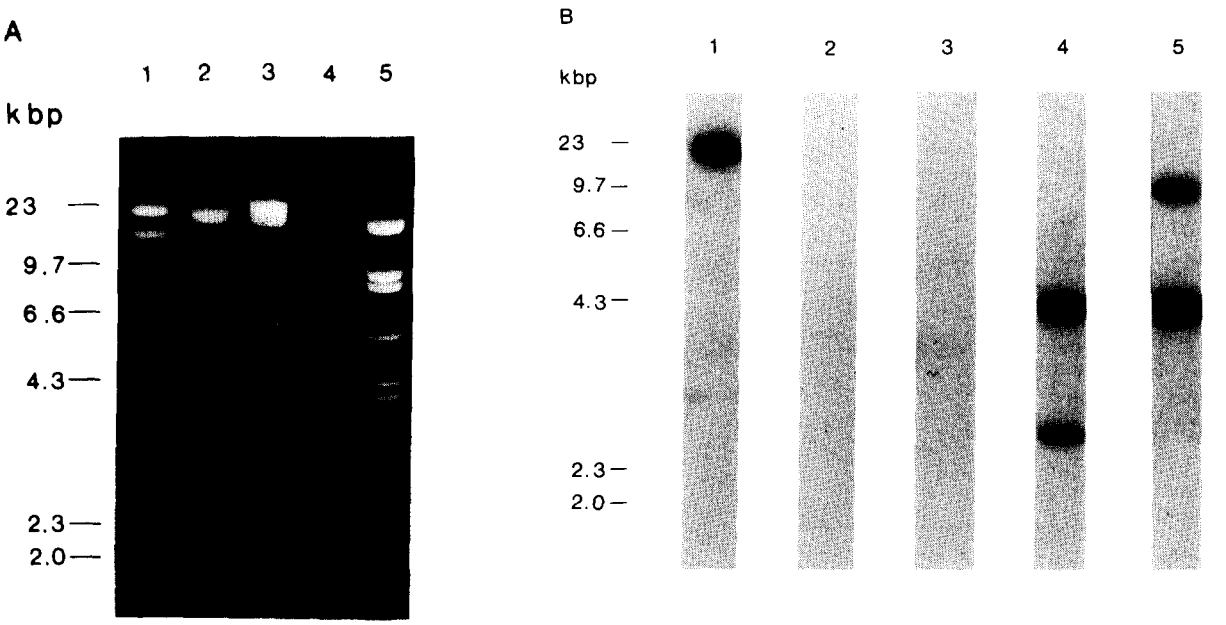

Fig. 2. Characterization of cosmid clones isolated from a human and feline cosmid gene library. (A) DNA of cosmid clones ALLW-1283-Cl21 (lane 1), MB10 (lane 2), MB25 (lane 3) and feline cosmid clones MB65 (lane 4) and MB70 (lane 5) was digested with restriction endonuclease *Hin*dIII (lanes 1, 2 and 3) or with *Eco*RI (lanes 4 and 5), size-fractionated by agarose gel electrophoresis (0.6% agarose gel) and visualized by ethidium bromide staining. (B) Southern blot analysis of human cosmid clones ALLW-1283-Cl21 (lane 1), MB10 (lane 2), MB25 (lane 3) and feline cosmid clones MB65 (lane 4) and MB70 (lane 5) with v-*sis*-specific molecular probes FAO1 and FAO2. Digestion with restriction endonucleases is as described under A. Cosmid clones MB10 and MB25 were isolated by 'chromosome walking' (see Fig. 3) using the insert of pAO79 as a probe. Molecular weight markers were as described in the legend to Fig. 1.

restriction endonuclease digestion patterns using the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Xba*I and *Xho*I or combinations of these in sequential digestions (data summarized in Fig. 3). The orientation of c-*sis* sequences of ALLW-1283-Cl21 was obtained by hybridization analysis using FAO1 or FAO2 as a 5′ or 3′ v-*sis*-specific probe (data summarized in Fig. 3).

For the purpose of 'chromosome walking', a unique 5′-specific (pAO79) and 3′-specific (pAO73) probe was prepared from clone ALLW-1283-Cl21 and used in further screening of the human cosmid library. Two positive clones, designated MB10 and MB25, were found (Fig. 2A, lanes 2 and 3, and Fig. 2B, lanes 2 and 3), both extending in a direction upstream of clone ALLW-1283-Cl21 (Fig. 3). The inserts of these cosmid clones did not hybridize with v-*sis* (Fig. 2B, lanes 2 and 3). No positive cosmid clones were found after screening another 200 000 colonies with pAO73.

In parallel experiments, two cosmid clones containing c-*sis* sequences were obtained upon screening of the feline cosmid library (500 000 colonies) with probes FAO1 and FAO2 (Fig. 2A, lanes 4 and 5; Fig. 2B, lanes 4 and 5). The complete feline v-*sis* cellular homolog was present in these cosmid clones. Determination of the orientation of feline c-*sis* and construction of its restriction enzyme map was done in the same way as described for the human c-*sis* clones (Fig. 3).

As summarized in Fig. 3, the three human cosmid clones contain overlapping sequences corresponding to a single contiguous region of human cellular DNA of about 65 kbp. The distribution of the v-*sis* homologous region is in accordance with previous reports [40–42] in which a total of six exons were identified. The feline DNA inserts represent about 60 kbp of contiguous cellular DNA and the organization of the v-*sis* homologous regions seems similar to those in the human locus.

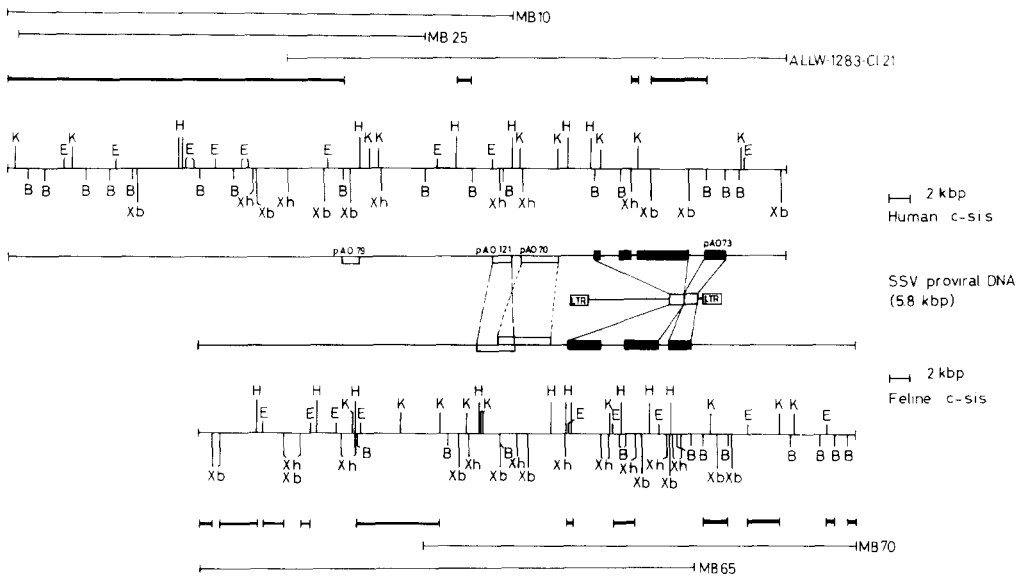

Fig. 3. Restriction endonuclease map of the human and feline c-*sis*-containing DNA regions. In the upper part of the figure, cellular DNA inserts of the human cosmid clones ALLW-1283-Cl21, MB10 and MB25 are shown. Directly below these inserts, solid bars indicate the distribution of highly repetitive DNA sequences detected in the human c-*sis* region. The next solid line represents a schematic restriction endonuclease map of the human c-*sis* containing DNA region. In this map, v-*sis* homologous genetic sequences are indicated as heavy bars. Homology with either half of the v-*sis* regions is indicated. Open boxes represent the DNA inserts of pAO70, pAO79 and pAO121, which were used as molecular probes. The DNA insert of pAO73 contains the 3′ v-*sis* homologous region and, therefore, is depicted as a heavy bar. The next line represents the SSV proviral genome. The open boxes are the long terminal repeats (LTRs). The 5′ and 3′ halves of the viral oncogene (v-*sis*) were subcloned in bacteriophage m13mp8 resulting in FAO1 and FAO2, respectively. The restriction endonuclease map of the feline c-*sis* proto-oncogene is shown under the SSV genome. Again, the genetic sequences homologous to v-*sis* are given as heavy bars. Their hybridization with the DNA inserts of FAO1 and FAO2 is specified. Feline DNA fragments containing genetic sequences homologous to the human DNA inserts of pAO70 and pAO121 are indicated as open boxes. The solid bars below the feline restriction endonuclease map represent highly repetitive DNA sequences within the feline c-*sis* region. The two solid lines at the bottom of the figure indicate the size and the relative position of the feline cellular DNA inserts within cosmid clones MB65 and MB70. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Xb, *Xba*I; Xh, *Xho*I.

Comparative analysis of the human and feline c-sis locus

As an approach to identify c-*sis*-specific coding sequences outside the v-*sis* homologous regions, a comparative analysis of the cosmid clones obtained from the human and feline gene libraries was performed. First of all, highly repetitive genetic sequences were identified. This mapping was performed by Southern blot analysis using a number of restriction endonucleases and with ^{32}P-labeled total human or feline DNA as a molecular probe (data not shown). The position of the repetitive sequences within the human and feline c-*sis*-containing DNA region was determined and the results are summarized in Fig. 3. As can be seen, relatively long stretches of nonrepetitive DNA are present upstream of the v-*sis* homologous regions of both species. To see whether both species share homologous genetic sequences in this area, a number of restriction enzyme digests of the human c-*sis* cosmid clone were hybridized with ^{32}P-labeled feline c-*sis* containing cosmid clones (data not shown). The results indicated that there were homologous genetic sequences in addition to the v-*sis* related sequences. These sequences were found in an *Eco*RI/*Hin*dIII DNA fragment of 2.0 kbp (pAO121) and a *Kpn*I/*Kpn*I DNA fragment of 3.8 kbp (pAO70), subcloned and used as probes in further studies (see Fig. 3). Southern blot analysis of human and feline genomic DNA with these probes indicated that they both represented unique DNA sequences (data not shown). Hybridization

analysis of the feline c-*sis* locus with probe pAO121 revealed a 3.5 kbp *Hin*dIII/*Xho*I hybridizing DNA fragment (Fig. 4A, lane 1). Upon digestion with restriction endonucleases *Bam*HI, *Hin*dIII and *Xho*I a 2.0 kbp and an 1.5 kbp hybridizing fragment were seen (Fig. 4A, lane 2). Hybridization of the feline c-*sis* clones with pAO70 yielded another *Hin*dIII/*Xho*I restriction fragment of 3.5 kbp (Fig. 4B, lane 1). Upon digestion of the feline cosmid clones with restriction endonucleases *Bam*HI, *Hin*dIII and *Xho*I, a 3.5 kbp and an 1.5 kbp hybridizing DNA fragment were detected (Fig. 4B, lane 2) (for localization of the DNA fragments, see Fig. 3). In Fig. 3, the identified homologous regions in the human and feline c-*sis* clones have been depicted. These results indicate the presence of unique and conserved genetic sequences in close proximity and upstream of the v-*sis* homologous region in the c-*sis* proto-oncogenes of both species.

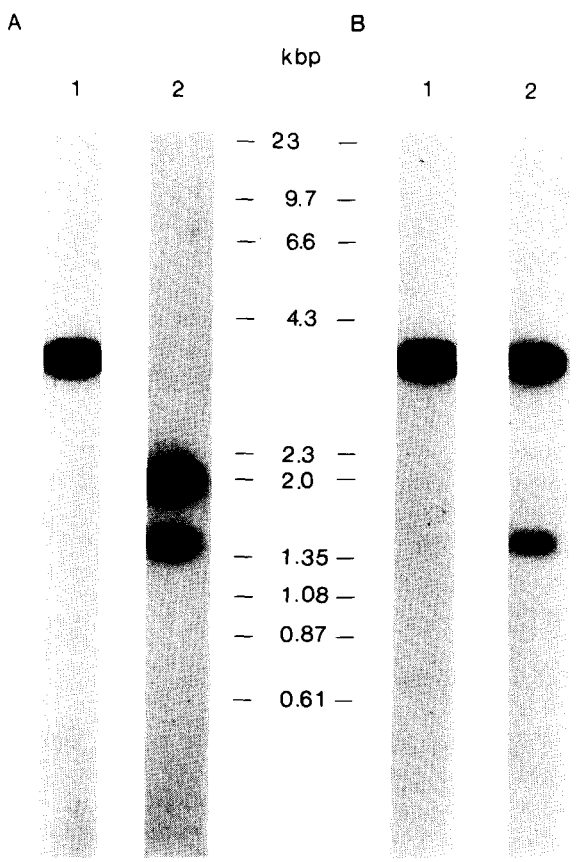

Fig. 4. Identification of unique genetic sequences in feline cosmid clone MB70 that are homologous to the human DNA inserts of pAO70 and pAO121. MB70 DNA (0.1 μg) was digested with restriction endonucleases *Hin*dIII and *Xho*I (A, lane 1; B, lane 1) or with *Bam*HI, *Hin*dIII and *Xho*I (A, lane 2; B, lane 2), electrophoresed through an 0.6% agarose gel, blotted onto nitrocellulose and hybridized to the ^{32}P-labeled 2.0 kbp *Eco*RI/*Hin*dIII insert of pAO121 (A) or 3.8 kbp *Kpn*I/*Kpn*I DNA insert of pAO70 (B). Molecular weight markers include λDNA digested with restriction endonuclease *Hin*dIII and DNA of φX174 digested with *Hae*III.

Northern blot analysis of human c-sis transcripts

The possibility that the additional homologous genetic sequences identified in the comparative analysis described above could represent a coding portion of the c-*sis* locus was tested in Northern blot analysis. Using a v-*sis*- (FAO1 and FAO2) and c-*sis*-specific (pAO73) probe, poly(A) selected RNA isolated from human lung tumor cells (lung carcinoma No. 4923) was shown to contain c-*sis* transcripts (Fig. 5, lanes 3, 4 and 5). No such transcripts could be detected in a similar analysis of RNA isolated from human cell line IARC-EW1 [43] (Fig. 5, lanes 1 and 2). With probe pAO121, the same 4.2 kbp transcript as detected with the v-*sis*- and c-*sis*-specific probes appeared to hybridize (Fig. 5, lane 6). These results strongly favor the possibility that some genetic sequences in the

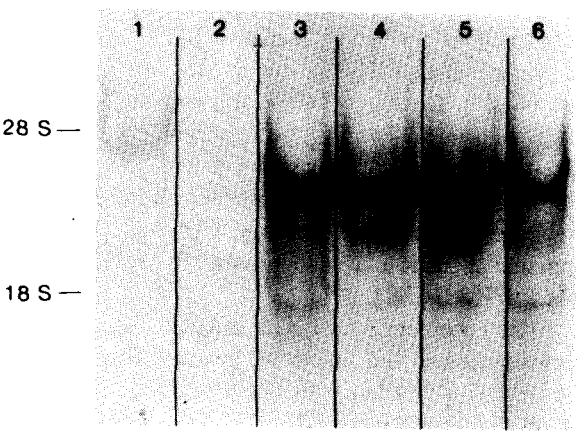

Fig. 5. Northern blot analysis of RNA transcripts in human lung tumor cells. Poly(A)-selected RNA samples were obtained from a human cell line (IARC-EW1) (lanes 1 and 2) and a human lung tumor (lung carcinoma No. 4923) (lanes 3, 4, 5 and 6) and screened (45-μg aliquots) in Northern blot analysis with the following *sis* probes: FAO1 (lanes 1 and 3), FAO2 (lanes 2 and 4), pAO73 (lane 5) and pA0121 (lane 6). Molecular weight markers included 28 S and 17 S ribosomal RNA.

2.0 kbp *Eco*RI/*Hin*dIII DNA fragment represent human c-*sis* coding sequences.

Discussion

In the present study, the v-*sis* homologous DNA sequences of human and cat have been studied by Southern blot analysis followed by molecular cloning of both *sis* cellular homologs. Southern blot analysis revealed that the v-*sis* homologous region in both species was rather extensive. Furthermore, a *Hin*dIII-RFLP was frequently detected in the human locus. Among human DNA samples from ten unrelated individuals, including five leukemic patients, six appeared to have an additional *Hin*dIII site in one allele. The other four were homozygous for the absence of this site. Interestingly, no individual was found to possess the *Hin*dIII site in both alleles. The additional *Hin*dIIII site could be localized in the middle of the v-*sis* homologous DNA region.

With the v-*sis* viral oncogene as a molecular probe, the c-*sis* containing DNA regions were isolated from cosmid gene libraries of the two species. A contiguous human DNA region of about 65 kbp was isolated and it appeared to contain all the human v-*sis* homologous sequences distributed discontinuously over a DNA region of approx. 12 kbp. The restriction endonuclease digestion data obtained in this study confirm data reported by Johnsson et al. [41], Chiu et al. [42] and Josephs et al. [44,45]. In these studies, the size and distribution of the six human v-*sis* homologous regions are described in detail. At the 3′ end, the presence of an untranslated region was reported and part of it appeared to lack homology with v-*sis* [45]. This particular region is also present in cosmid clone ALLW-1283-Cl21.

The complete feline c-*sis* cellular homolog was represented in the inserts of cosmid clones MB65 and MB70. The feline v-*sis* homologous sequences were dispersed over approx. 12 kbp of the 60 kbp contiguous feline DNA region isolated. Upon comparative hybridization analysis of the human c-*sis* cosmid clone ALLW-1283-Cl21 and the two feline clones, the existence of common and highly conserved genetic sequences in addition to the v-*sis* homologous regions became apparent. Northern blot analysis established that a transcript of the c-*sis* proto-oncogene of man contained one of these sequences. These results indicate that the *sis* proto-oncogene contains as yet unidentified coding sequences that map upstream of the region that was captured in the viral oncogene of SSV. Indications for the concept that the c-*sis* locus contains additional coding sequences and that SSV acquired only a portion of this gene could also be deduced from the size difference of human c-*sis* transcripts [26,27] and the v-*sis* oncogene [23,24]. DNA sequence analysis of the v-*sis* homologous DNA region in the human locus revealed that the first exon lacked an initiation codon, again indicating the presence of additional exon sequences at the 5′ end of c-*sis*.

A cDNA clone, designated pSM-1, was described by others [46]. This clone was obtained from mRNA isolated from HUT-102 cells and represented a truncated form of the c-*sis* mRNA. About 2.7 kbp of 3′ c-*sis* coding sequences appeared to be present in pSM-1 and an initiation codon was found about 64 bp upstream of the v-*sis* homologous region. Transfection of pSM-1 into NIH-3T3 cells resulted in morphological transformation, while genomic DNA isolated from HUT-102 cells failed to do the same. These authors speculated that the upstream c-*sis* genetic sequences might code for regulatory domains of PDGF, the putative gene product of the c-*sis* gene. The newly discovered c-*sis* exon described in this report might be instrumental in verifying their speculation.

Acknowledgements

We thank A.J.M. Roebroek for helpful discussions and preparation of molecular probes. This work was supported by the Netherlands Cancer Foundation, Contract No. NUKC Bioch. 80-1.

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