
The repressor sequence upstream of *c-mos* acts neither as polyadenylation site nor as transcription termination region

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Received 5 September 1986; Revised and Accepted 21 October 1986

ABSTRACT

Recently we reported that the *c-mos*(rat) coding region is preceded by sequences (RIS) which repress accumulation of *c-mos* RNA in the cytoplasm. To investigate the effect of RIS on RNA transcription or processing a retroviral promoter was inserted at different positions relative to RIS. Cotransfection was used to establish cell lines with high copy number of the plasmids and to avoid any selection for *c-mos* expression or RIS function. Analysis of RNA in the cell lines indicated that RIS does not provide a poly(A) site and allowed characterization of the *c-mos*(rat) poly(A) site. Surprisingly, RIS contains sequences homologous to elements involved in eucaryotic RNA cleavage/polyadenylation. To determine an effect of RIS on transcription, RNA was elongated *in vitro* in nuclei isolated from the cell lines and used to analyze the number of RNA polymerase II molecules transcribing different regions of the plasmid. The analysis showed that RIS does not act as transcription termination region.

INTRODUCTION

The *c-mos* gene is expressed in rat and mouse testis, ovary and embryos in which RNA species of 1.7, 1.4 and 6.0 kb respectively were detected (1). The repression of expression in all other tissues and cell lines examined (2) suggested the existence of sequences which repress *c-mos*. We (3) and others (4) recently reported evidence for such sequences in rat and mouse, called RIS and UMS respectively, which act as repressor when located downstream of a promoter. We showed that the RIS element is located approximately 1.8 kb upstream of the *c-mos*(rat) coding region and that it represses the accumulation of *c-mos* RNA in the cytoplasm in transient transfection assays (3). Wood et al. (4) reported the presence of consensus sequences involved in eucaryotic polyadenylation in UMS. This suggested that RIS acts through one or several of the following mechanisms: a) RIS contains a poly(A) site causing cleavage/polyadenylation of larger precursor RNAs, b) RIS is a transcription termination region in which RNA polymerase II molecules are released from the

DNA template and c) RIS might contain sequences which destabilize RNA molecules leading to their rapid degradation.

For several eucaryotic protein coding genes the region in which transcription termination occurs has been analyzed (5). It became evident that termination of transcription by RNA polymerase II does not occur at a particular site, but rather in a region ranging in size from 170 to 1210 bp (5,6,7). These regions are located from several hundred to a few thousand bp downstream of the poly(A) site (5). Recently, evidence for the involvement of particular sequences in the termination process was reported (9). For cleavage/polyadenylation of RNA transcribed from eucaryotic protein coding genes two sequence motifs were found: the AATAAA box (10) and the TG box (11,12). Recently, the function of these elements in the cleavage of the RNA precursor and in the addition of the poly(A) tail were separately analyzed *in vitro* (13,14,15). A function of the RIS element as termination region or as poly(A) site could account for the significantly reduced levels of *c-mos* RNA in the cytoplasm.

Both possibilities were investigated using plasmids which contain a retroviral long terminal repeat (LTR) promoter inserted upstream of RIS or lacking RIS. Size analysis and S₁ nuclease mapping of RNA transcribed from the plasmids were used to determine the amount of *c-mos* RNA and the location of the poly(A) site(s). Nuclei were isolated from cell lines containing high copy numbers of the plasmids and used for *in vitro* elongation of RNA (16). Nascent RNA was hybridized to filter-bound single stranded DNA, containing selected regions of the plasmids. The nucleotide sequence of the *c-mos*(rat) poly(A) site and of part of RIS was determined.

MATERIALS AND METHODS

Cells and transfection

LTK⁻ cells were grown in α -Minimal Essential Medium supplemented with 10 % fetal calf serum (Gibco). Cells were transfected with plasmid DNAs by the calcium phosphate coprecipitation method (17) and selected for HAT-resistance as described before (18).

DNA constructs

Plasmids pSK and pXK were derived from plasmid pREK. pREK harbors the 5.5 kb EcoR I-Kpn I fragment isolated from λ .D₃e (19) which contains *c-mos*(rat) (Fig.1) inserted into the EcoR I-Pvu II sites of pBR322. pSK was generated by substituting the 0.75 kb EcoR I-Sma I rat DNA fragment of pREK by the 0.6 kb EcoR I-Sma I fragment isolated from pMLTR (20), which contains the

LTR of Moloney sarcoma virus (Fig.1). pXK was generated by substituting the 3.1 kb EcoR I-Xba I rat DNA fragment of pREK by the LTR-containing 0.6 kb EcoR I-Sma I fragment (Fig.1). The RIS element is present in pSK, but not in pXK. pMLVCH (Fig.1) was described before (21) and contains a hybrid mos gene inserted into the Sma I site of pMLTR: the hybrid gene consists of the N-terminal 0.5 kb of v-mos linked at the unique Kpn I site to the C-terminal 0.6 kb of c-mos(rat) and 0.65 kb of 3' flanking sequences. Thus pMLVCH contains the potential c-mos poly(A) site as well as a downstream LTR poly(A) site (Fig.1).

Bacteriophage M13 clones A, B, C and D (Fig.4): clone A harbors the 0.31 kb Xho I-Pst I fragment representing sequences located upstream of RIS, clone B includes the 0.55 kb Xho I-Pvu II fragment representing RIS, clone C harbors the 0.4 kb Hind III fragment which contains the C-terminal c-mos coding region, clone D harbors the 0.6 kb Hind III-Kpn I fragment representing sequences downstream of c-mos.

DNA and RNA analysis

Cellular DNA was isolated and analyzed by Southern blotting analysis as described before (20), using a nick-translated 0.6 kb Xho I-Sac I DNA fragment (Fig.1) (19) containing c-mos coding sequences as probe in the hybridizations. Restriction enzymes were used as prescribed by the manufacturer (Boehringer). Total cytoplasmic RNA was isolated using standard methods (22). Size analysis of RNA was performed by Northern blotting (20) using the c-mos specific probe described above as well as a nick-translated 2.35 kb Sma I-Xba I DNA fragment containing c-mos upstream sequences. The latter probe only detects pSK specific RNA while the c-mos specific probe detects both pSK and pXK specific RNAs. S₁ nuclease mapping of poly(A) site(s) was performed as described (23) using the c-mos specific probe to detect protected fragments.

DNA nucleotide sequence analysis was done by the dideoxy chain termination method as described (19).

In vitro elongation of nascent RNA in isolated nuclei and hybridization

Nuclei were isolated from 50×10^6 LTK⁻ cells and L-pSK cells and elongation reactions were done as described by Schibler et al. (16). Purified, in vitro elongated labeled RNA (approximate size 160 N) was used in hybridizations as described by Hagenbüchle et al. (6), except that the prewash time and the hybridization time of dot-blot filters were 2 and 24 hr respectively. Probes spotted on filters were single strand DNA from bacteriophage M13 clones A, B, C and D, single strand M13 DNA and denatured β -actin-specific DNA (pAL41 (24)).

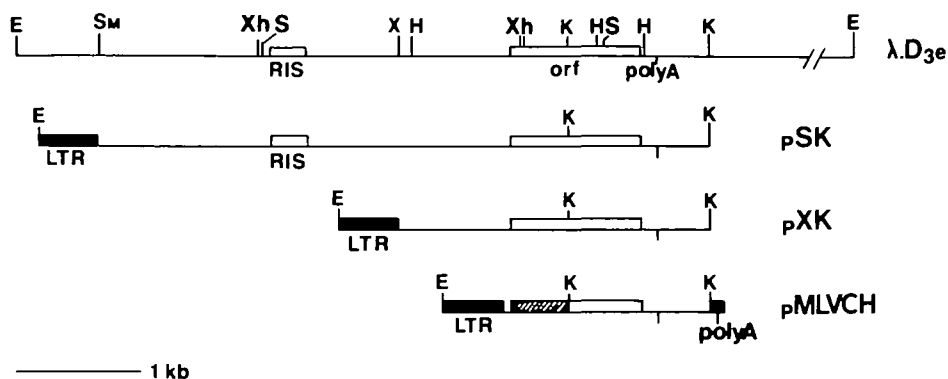


Figure 1. Schematic representation of constructs. To study the activity exerted by the repressor sequences (RIS) the long terminal repeat (LTR, black box) of Moloney sarcoma virus was introduced in λ .D_{3e} DNA (20) at the indicated positions. The white box denotes the *c-mos*(rat) coding region (orf) and the indicated position of the *c-mos*(rat) poly(A) site was determined in this study. Also indicated is the second poly(A) site in plasmid pMLVCH-1. The hatched box represents *v-mos* sequences. E=EcoR I, H=Hind III, K=Kpn I, S=Sac I, Sm=Sma I, X=Xba I and Xh=Xho I.

RESULTS

RIS inhibits RNA accumulation but does not act as poly(A) site

To investigate if RIS acts by providing a poly(A) site and to characterize the *c-mos*(rat) poly(A) site constructs were made as described in Materials and Methods, which are schematically depicted in Fig.1. The approximate location of RIS and the location of the *c-mos* coding region (orf) are indicated. Preliminary sequence analysis had shown the presence of a potentially functional poly(A) site downstream of orf as indicated. The positions of the LTR with respect to RIS and orf are shown. The distance between the LTR and RIS in plasmid pSK is 1.6 to 1.8 kb. pMLVCH contains both the putative *c-mos* poly(A) site and the functional retroviral poly(A) site located 0.6 kb downstream (21,24). The latter allows detection of stable *mos* RNA if the *c-mos* poly(A) site were not functional.

To establish cell lines containing high copy numbers of the plasmids pSK and pXK and to avoid any possible selective pressure on *c-mos* expression or RIS activity, LTK⁻ cells were cotransfected with the plasmids and the Herpes simplex virus thymidine kinase (TK) gene. Pools of HAT-resistant colonies, called L-pSK and L-pXK, were grown for analyses. Mouse fibroblasts containing pMLVCH DNA (C3H-pMLVCH) have been described before (21). To determine the number of integrated, unrearranged copies of the plasmids cellular DNA isolated from L-pSK and L-pXK cells was analyzed by restriction with the

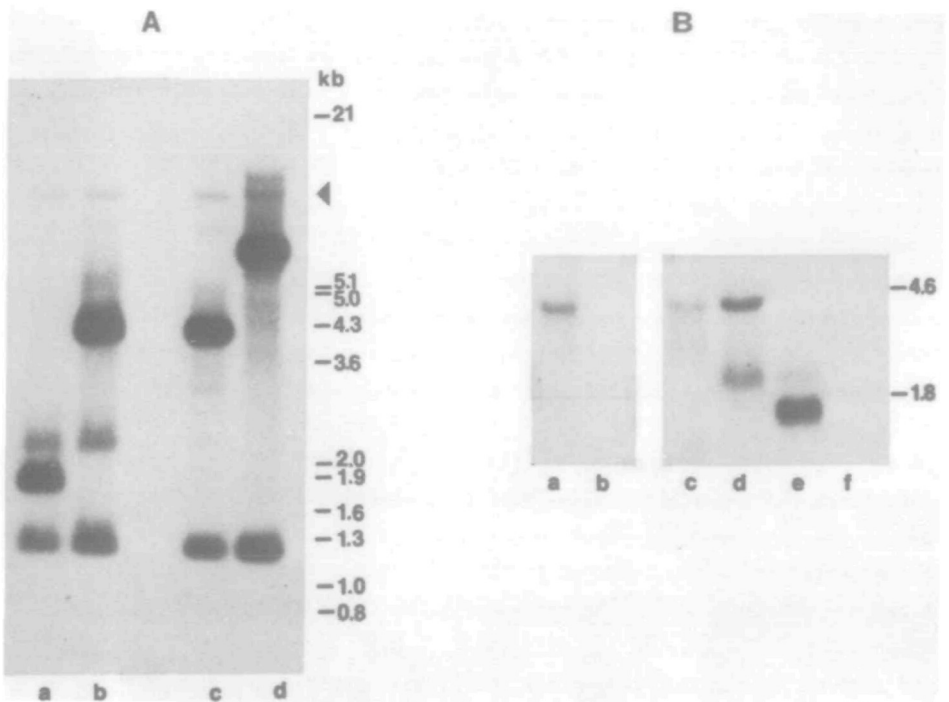


Figure 2. Integration and expression of pSK and pXK plasmids in LTK⁻ cells. L-pSK and L-pXK cells were established by cotransfection of the HSV TK gene and pSK and pXK DNA. A) Cellular DNA isolated from L-pXK (lanes a and c) and L-pSK (lanes b and d) was digested with EcoR I-Kpn I (lanes a and b) or Kpn I (lanes c and d) and analyzed by Southern hybridization using a c-mos specific probe. The arrow indicates the 11 kb Kpn I c-mos(mouse) fragment. B) Total cytoplasmic RNA isolated from L-pSK (lanes a and c), L-pXK (lanes b and d), C3H-pMLVCH (lane e) and LTK⁻ (lane f) was analyzed by Northern hybridizations using the upstream mos probe (lanes a and b) or the c-mos specific probe (lanes c-f). Ribosomal RNAs served as markers.

enzymes EcoR I-Kpn I and Kpn I alone (Figs.1 and 2). L cells harbor one copy of c-mos(mouse), which generates fragments of 11 and 1.3 kb upon digestion with Kpn I or with EcoR I-Kpn I (23). These fragments served as internal standards in the determination of the plasmid copy number. The result of the DNA analysis is shown in Fig.2A. The 1.8 and 1.25 kb EcoR I-Kpn I fragments expected for pXK (Fig.1) and the 4.2 and 1.25 kb EcoR I-Kpn I fragments expected for pSK (Fig.1) were detected with the mos specific probe (Fig.2A, lanes a and b), indicating that the integrated plasmid DNAs do not contain major rearrangements. The detection of the 4.1 and 1.25 kb Kpn I fragments

expected for pXK (lane c) and of the 6.5 and 1.25 kb Kpn I fragments expected for pSK (lane d) indicated that the plasmid DNAs are tandemly integrated. The 11 kb Kpn I c-mos(mouse) fragment is indicated by the arrow. The 1.3 kb Kpn I c-mos(mouse) fragment is not detectable in this analysis due to the large amounts of the plasmid-specific 1.25 kb fragment. Densitometry of the signals originating from the 11 kb fragment and from the plasmid-specific fragments showed the presence of 30-40 copies of plasmid pXK in L-pXK cells and of 70-80 copies of plasmid pSK in L-pSK cells.

The cell lines described above were used for RNA analyses. If RIS provides a functional poly(A) site for precursor RNAs initiated in the LTR in pSK, then a cytoplasmic transcript of approximately 1.7 kb should be detectable using the 2.35 kb Sma I-Xba I upstream mos fragment as probe (Fig.1; Materials and Methods). If only the putative c-mos poly(A) site is functional then a 4.3 kb pSK-specific transcript should be detectable using either the upstream mos probe or a c-mos specific probe. In this latter case the expected size of pXK-specific RNA in L-pXK cells is 2.0 kb. The expected sizes of pMLVCH-specific RNA in C3H-pMLVCH cells are 1.5 and/or 2.0 kb depending on the poly(A) site(s) used. Fig.2B gives the results of the size analysis of total cytoplasmic RNA. L-pSK cells express the 4.3 kb RNA species, which is detectable with both the upstream mos probe (lane a) and the mos-specific probe (lane c). No other pSK-specific transcripts were detected. L-pXK cells express RNA species of 2.0 kb and 4.4 kb, which are detectable with the mos-specific probe (lane d), but not with the upstream mos probe (lane b). The use of additional probes demonstrated that the 4.4 kb species is a read-through transcript terminating close to the pBR322 EcoR I site, at which position a consensus AATAAA box is present (26) (not shown). C3H-pMLVCH cells express the 1.5 kb RNA species as well as small amounts of the 2.0 kb RNA species (lane e) while LTK⁻ cells do not express mos-specific RNA (lane f). The analysis of poly(A)⁺ RNA isolated from all cell lines gave identical results (not shown), indicating that all RNA species detected are polyadenylated. We conclude a) that RIS does not contain a poly(A) site which acts efficiently enough to cause accumulation of polyadenylated RNA species in the cytoplasm and b) that the putative c-mos(rat) poly(A) site is functional (see below). Densitometry of the exposures shown indicated that the level of mos RNA transcripts in stably transfected L-pSK cells is 2.5 to 5 % of that in L-pXK cells after correction for the different plasmid copy numbers. This value is in close agreement with data we obtained previously using transient transfection assays (3).

The c-mos(rat) poly(A) site

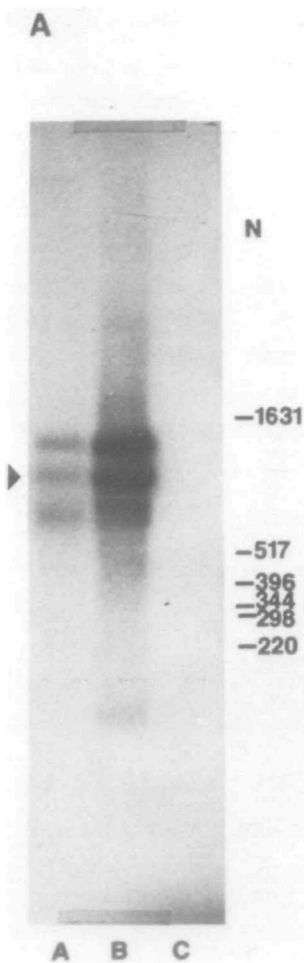
The sizes of RNA transcripts in L-pSK, L-pXK and C3H-pMLVCH cells suggested the presence of a functional c-mos(rat) poly(A) site downstream of orf. This site was characterized by S_1 nuclease mapping and nucleotide sequence analysis. RNA from L-pSK, L-pXK and LTK⁻ cells was hybridized to a 1.5 kb DNA fragment which spans the region from the c-mos(rat) Xho I site to the right Kpn I site (Fig.1). After S_1 nuclease digestion the protected fragments were denatured with DMSO/glyoxal, separated on agarose gels and transferred to nitrocellulose filters (22,23). The mos-specific probe was used in the filter hybridizations and the result is shown in Fig.3A. RNA from L-pSK cells (lane a) and L-pXK cells (lane b), but not from LTK⁻ cells, protected a 1.1 kb fragment from S_1 digestion (arrow), which indicates the presence of a poly(A) site approximately 80 bp downstream of the orf stop codon (19). The 1.5 kb DNA fragment used in the hybridizations is also detected. The nucleotide sequence analysis of this region, which is presented in Fig.3B, demonstrates the presence of the AATAAA box (N 1269-1274) and of the TG box (N 1372-1378), both of which were shown to be implicated in the cleavage/polyadenylation steps of RNA polymerase II transcripts (5). The comparison with the published c-mos(mouse) sequence (24) indicated a high degree of homology in the 3' untranslated region with the exception of a 14 bp deletion in rat DNA.

Part of RIS was sequenced to compare to the published UMS sequence (4) as shown in Fig.3C. Surprisingly, this analysis revealed the presence of an AATAAA box and of a TG box both of which are conserved between mouse (4) and rat. No other particular features were found by computer analysis of the sequence. We conclude that the two sequence elements implicated in cleavage/polyadenylation, which are present both in RIS and downstream of c-mos(rat), are only functional at the c-mos(rat) poly(A) site (see Discussion).

RIS does not function as transcription termination region

The possibility of transcription termination in RIS was investigated using the L-pSK cells, which harbor 70 to 80 copies of plasmid pSK. Such an activity might help explain the greatly reduced amounts of plasmid-specific RNA in L-pSK cells.

Nuclei were isolated from L-pSK cells and from LTK⁻ cells. Preliminary experiments had indicated that the elongation rate of *in vitro* transcribed RNA in the nuclei was linear over a period of 15 min. No pSK-specific RNA was transcribed in the presence of α -amanitin (not shown). The elongation of labeled RNA was allowed to proceed for 10 min and the labeled, nascent RNA was



B

Hind III

1260

rat mouse AAGCTTTTCGTTTCTGTTTATTTT--AAATAAGTAAGGATGGGCTTTT-----AGGGCATAT

T C TT G TGTGGAGAAAAACATACCACT

poly-A

1330

TTTTAGAAAATAAAGTTACTACAAACTTCAGCCCTGAAGTGCCTTTCCTAAGACTAACGAGGGTACAGATATC

G C H

TG

1400

ATGGGCAACCACTCAGGTTGTGTTAAACAACCTGAGCCCTTTGTGTGTCACCTTTCTGGGTCTACTGTATCATT

1440

TCCGTAGTAAGGTTTGTTCCTGAAATGAAAAGAGAAAGGGGGT

C

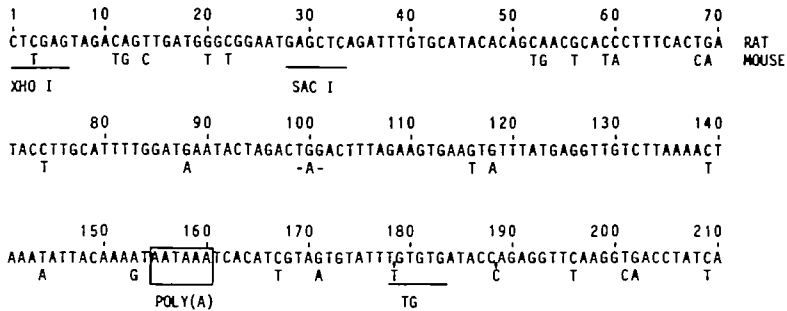


Figure 3. Characterization of the c-mos(rat) poly(A) site. A) S_1 nuclease mapping of the 3' end of plasmid specific RNAs: a 1.5 kb Xho I-Kpn I fragment covering most of c-mos(rat) and its downstream flanking sequences (Fig.1) was hybridized to total cytoplasmic RNA from L-pSK (lane A), L-pXK (lane B) and LTK⁻ (lane C) cells. After S_1 digestion the protected fragments were denatured and analyzed by Northern hybridization using a c-mos specific probe. A Hinf I digest of pBR322 DNA served as marker. B) Nucleotide sequence analysis of the region containing the functional c-mos(rat) poly(A) site, starting at the right Hind III site which is located 24 bp downstream of the c-mos(rat) stop codon (19). The rat sequence is compared to the c-mos(mouse) sequence (25) of which the last published nucleotide is indicated by the arrow. The numbering of nucleotides is according to (19). Only nucleotide differences and deletions are indicated. Also indicated are the AATAAA box and a sequence homologous to the TG box. C) Part of the RIS element, which was tested for its capacity to function as poly(A) site, was sequenced. Indicated are the Xho I and Sac I restriction sites (Fig.1), as well as the AATAAA box and a sequence homologous to the TG box. The rat sequence is compared to the c-mos(mouse) upstream element UMS (4) and nucleotide differences, deletions and insertions are indicated.

purified. To examine the density of RNA polymerase II molecules on pSK DNA in L-pSK cells, 4 fragments were isolated from pSK and subcloned in the bacteriophage M13 such that the M13 + strands contained the non-transcribed pSK strands (Fig.4): clone A is specific for the region upstream of RIS and was included as a measure for the number of RNA polymerase II molecules initiated in the LTR of pSK, clone B contains RIS, clone C is specific for the C-terminal c-mos(rat) part and clone D contains the c-mos(rat) poly(A) site and downstream sequences (Fig.4). If RIS acts as transcription termination region the density of polymerase molecules on the sequences represented in clones C and D is expected to be very low or not detectable. Also included on the dot-blot filters were M13 + strand DNA and denatured β -actin DNA, which served as negative and positive controls respectively in the hybridization

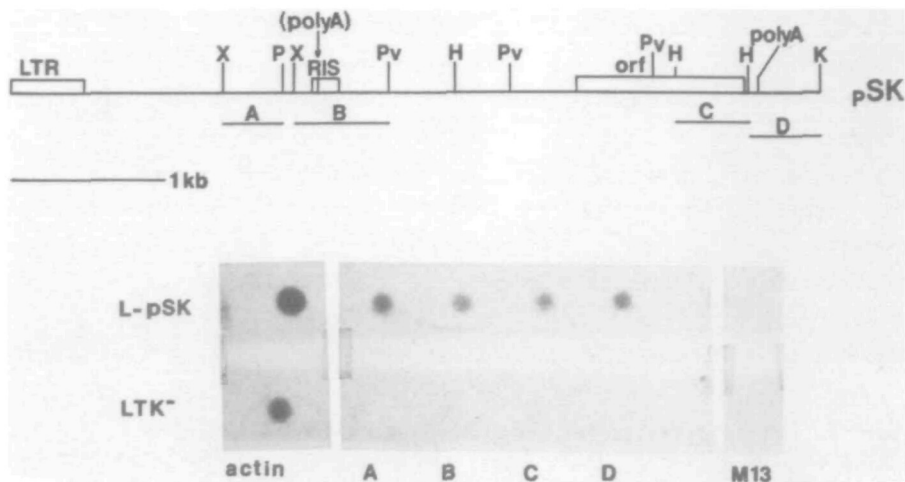


Figure 4. Transcription termination analysis in L-pSK cells. RNA was elongated *in vitro* in nuclei isolated from L-pSK cells and LTK⁻ cells and hybridized to dot-blot containing DNA from the bacteriophage M 13 clones A to D, of which the origin is indicated in the drawing representing plasmid pSK. M13 + strand DNA and denatured β -actin DNA were included on the dot-blot as negative and positive controls respectively. Indicated in the drawing are the LTR and orf (white boxes), the functional c-mos(rat) poly(A) site and the cryptic poly(A) site in RIS.

with the labeled, nascent RNA. The results of the hybridizations are shown in Fig.4. Both LTK⁻ and L-pSK cells actively transcribed the β -actin gene and do not express RNA species homologous to M13 DNA. No transcription of mouse sequences homologous to pSK DNA is detected in LTK⁻ cells. Comparison of the hybridization signals for clones A to D using L-pSK nascent RNA indicated an equal rate of transcription of pSK DNA in L-pSK cells in a region over 4 kb in length (Fig.4). From this we conclude that RIS is not a transcription termination region.

DISCUSSION

The mechanism of action of the upstream c-mos(rat) element RIS, which represses RNA accumulation (3), was investigated using different constructs which harbored an LTR promoter upstream or downstream of the RIS element. The RIS element is unusual in that it does not exert a cis-effect on downstream located promoters: we (3) and others (4) have previously shown that repression of RNA accumulation by RIS is only apparent if the promoter used is located upstream of RIS. Here we extend our observations by showing that RIS does not

act as a poly(A) site: L-pSK cells, which contain 70 to 80 unrearranged copies of plasmid pSK, express a 4.3 kb polyadenylated RNA species, indicative of a functional c-mos(rat) poly(A) site, but no 1.7 kb RNA species (neither polyadenylated nor poly(A)⁻) which would have been indicative of a poly(A) site within RIS. However, nucleotide sequence analysis of part of RIS demonstrated the presence of two sequence motifs, the AATAAA- (10) and TG-boxes (11,12), shown before to be implicated in many cases in the cleavage and polyadenylation steps of eucaryotic RNA polymerase II transcripts. Recent studies demonstrated that the cleavage step and polyadenylation step are separate events (14,15) and can be studied separately *in vitro* (13): Zarkower et al. (15) showed that the AATAAA box is required for cleavage, but not for polyadenylation of precleaved RNA *in vitro*. The role of the TG box is less clear: it also plays a role, although less important than the AATAAA box, in the cleavage step (15). RIS contains both motifs and it will be important to test *in vitro* if the presence of these signals result in cleavage of an *in vitro* prepared precursor RNA. If RIS acts as cleavage site, resulting in the synthesis of poly(A)⁻ transcripts, then conceivably such transcripts are unstable and therefore not detected in our assay, leading to the observed effect of RIS on RNA accumulation. However, another, more likely, possibility is the destabilization of mRNA by sequences in RIS. Evidence for such an activity exerted by particular sequences was recently reported by Shaw and Kamen (27): they showed that AU rich sequences, which are present in many transiently expressed genes, confer instability on heterologous RNA. Indeed, RIS and UMS (4) are very AT rich and it will be important to test this possibility.

The high plasmid copy number in L-pSK cells also allowed investigation whether RIS acts as transcription termination region of RNA polymerase II molecules initiated in the LTR of pSK. *In vitro* elongated, nascent RNA, synthesized in nuclei isolated from L-pSK cells and LTK⁻ cells, was hybridized to dot-blots containing single-stranded DNA probes representing different regions of pSK. The hybridization signals obtained showed that the density of RNA polymerase II molecules was equal in the region upstream of RIS (Fig.4, clone A) and in the region located 3 kb downstream of RIS (Fig.4, clone D). Therefore, the repression exerted by RIS is not at the level of RNA synthesis, but at the level of RNA processing. This analysis also indicated that the region upstream of RIS (present in clone A), which is homologous between rat and mouse as determined by hybridization, is not transcribed to the level of the β -actin gene in LTK⁻ mouse cells, but we cannot exclude a low level of

transcription. This is in agreement with the fact that no RNA species transcribed from this region were detected (Fig.2B, lane b).

Based on earlier data it was suggested that the *c-mos*(mouse) promoter is probably located upstream of the repressor sequences UMS (4). Combined with the data showing tissue-specific expression of *c-mos* (1), this suggests that the RIS and UMS repressor sequences might play a role in the determination of the tissue-specificity, possibly together with as yet unknown transcription elements: the repressor element might be inactivated in organs showing *c-mos* expression such as testis and ovary, and it might be active in all other tissues and organs. Our data which demonstrate that RIS functions at the level of RNA processing, possibly by destabilizing mRNA, provides a basis for studying the interaction between tissue-specific proteins and the repressor sequences.

ACKNOWLEDGMENTS

We thank Dr. Diggelmann in whose laboratory this work was performed for critical reading of the manuscript. F.A.v.d.H. was the recipient of an ISREC fellowship. This work was supported by a grant from the Swiss National Science Foundation.

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