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Transcription and replication silencer element is present within conserved region of human Alu repeats interacting with nuclear protein

Nikolai V. Tomilin, Sanae M.M. Iguchi-Ariga* and Hiroyoshi Ariga*

Institute of Cytology, The Academy of Sciences of the USSR, 194064 Leningrad USSR and **Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan*

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Human cells contain a nuclear protein interacting with Alu repeats, and this protein seems to recognize a conserved sequence motif, GGAGGC, present within the RNA polymerase III promoter and within the SV40 T-antigen-dependent ARS-like element. To study the potential functional role of this element, we have inserted the sequence into a chloramphenicolacetyltransferase (CAT) expression vector with a SV40 promoter and enhancer element from the up-stream region of the human *c-myc* gene, and transfected HeLa cells with the resulting plasmid. Analysis of expression by the CAT assay indicates that the Alu-derived sequence supresses transcription of the CAT gene driven by the *c-myc* enhancer/SV40 promoter. The Alu-derived sequence also inhibits ARS activity of the *c-myc* enhancer. The data allow the explaination of the transcriptional inactivity of Alu repeats in HeLa cells, and suggest the existence of a negative control of Alu transcription.

Alu sequence element; Chloramphenicolacetyltransferase assay; Silencer

1. INTRODUCTION

Human retroposons, the Alu family DNA repeats, are effectively transcribed by RNA polymerase III in the cytoplasmic S100 fraction from HeLa cells in vitro [1], but most of the Alu repeats are transcriptionally silent in HeLa cells in vivo [2], suggesting the existence of a nuclear repressor of Alu transcription in human cells. In our previous reports, we have described a nuclear protein from HeLa cells interacting with Alu repeats [3–5] and have located a major binding site for this protein within the 35 bp restriction subfragment of Alu [5] containing a T-antigen-dependent ARS-like element [6,7]. Experiments with short synthetic oligonucleotides [4] suggest that the GGAGGC sequence, which is conserved in Alu repeats [8], is important for the interaction of Alu binding protein with Alu and, therefore, may play a role in the control of Alu transcription. In this study, we have attempted to get direct experimental evidence for this hypothesis.

2. MATERIALS AND METHODS

Methods of calcium phosphate transfection of mammalian cells, preparation of nuclear extracts, DNA labeling, mobility shift and CAT (chloramphenicolacetyltransferase) assays were described before [4,5,9], and some details are given in the legends to figures. The sequence containing the GGAGGC box (Alu A oligonucleotide, see [4]) was inserted into the CAT expression vector p myc-o-pCAT [9] just upstream of the *c-myc* gene regulatory element (MRE) as shown in Fig. 1. CAT-free plasmid p AluA-myc-o was prepared in the same way except that the vector plasmid was p myc-o [9].

Both enhancer and ARS activities of MRE depend on the intracellular quantity of the *c-myc* protein [9] which is low in HeLa cells. To supply additional *c-myc* protein in trans, we have co-transfected the CAT plasmid in some experiments, with another plasmid (pSRalpha-myc) which expresses *c-myc* under the control of the retroviral promoter independently of the level of *c-myc* protein [11].

Correspondence address: N.V. Tomilin, Institute of Cytology, The Academy of Sciences of the USSR, 194064 Leningrad, USSR

3. RESULTS AND DISCUSSION

Fig. 2 shows the results of two independent experiments when CAT activity was measured two days after transfection of HeLa cells with the indicated plasmids. It is seen that both in the absence of additional *c-myc* protein (pUC18 carrier DNA, lanes 2 and 3 in A, lanes 1 and 2 in B) and in the presence of the protein in *trans* (pSRalpha-myc carrier DNA, lanes 4 and 5 in A, and lanes 3 and 4 in B) the AluA sequence inhibits CAT expression driven by the MRE enhancer-SV40 promoter. In the absence of additional *c-myc* protein provided in *trans*, the residual level of CAT expression was about the same as was observed with the enhancer-free plasmid pSVpCAT (Fig. 2B, compare lanes 2 and 5). It is also seen (Fig. 2) that the *trans*-activating effect of the pSRalpha-myc plasmid on the MRE enhancer varies significantly in the two experiments, and that the MRE activity in HeLa cells is lower compared to the SV40 enhancer (Fig. 2, lanes 1 and 4 in A) even in the presence of *c-myc* protein provided in *trans*. An inhibiting effect of the Alu-derived sequence on CAT expression driven by the MRE-enhancer/SV40 promoter was observed in 4 indepen-

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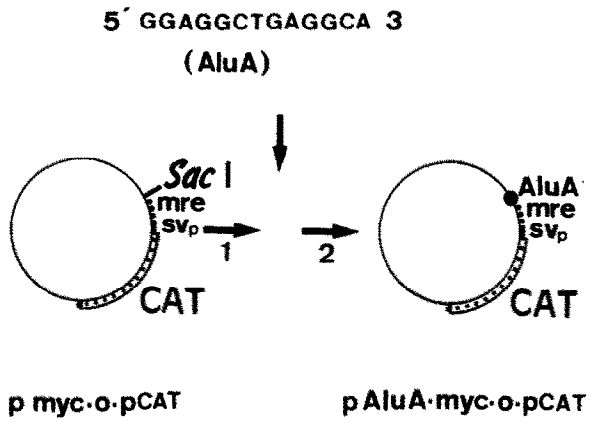

Fig. 1. Insertion of the conserved sequence of Alu repeats (AluA) into a CAT expression vector carrying the human *c-myc* gene upstream regulatory element (MRE). The pUC18-based plasmid p myc-o-pCAT (left) carrying MRE (21-mer, 5′ TCTCTTATGCGGTTGAATAGT 3′, see [9]), the enhancer-free SV40 promoter (SVp) and the chloramphenicolacetyltransferase (CAT) gene was cleaved with *Sac*I, treated with T4 phage DNA polymerase in the presence of dNTPs to create blunt ends (step 1), and ligated (step 2) to 5′-phosphorylated (with polynucleotide kinase) annealed synthetic oligonucleotides AluA1 and AluA2 [4] which form AluA. The upper strand of AluA (AluA1) is shown on the figure, the lower strand is 5′ TGCCTCAGCCTCC 3′. The presence of the AluA insert in the resulting plasmid (right, p AluA-myc-o-pCAT) was confirmed by hybridization, restriction mapping and sequencing (Sanger's method). The AluA sequence corresponds to nucleotides 177–189 of the consensus sequence of the Alu family [8]. The orientation of the AluA insert in the obtained plasmid corresponds to the upper strand (clockwise), and two tandem copies are present. AluA1 and A2 oligonucleotides were synthesized using the Gene Assembler (Pharmacia), and their purity was checked by polyacrylamide gel electrophoresis.

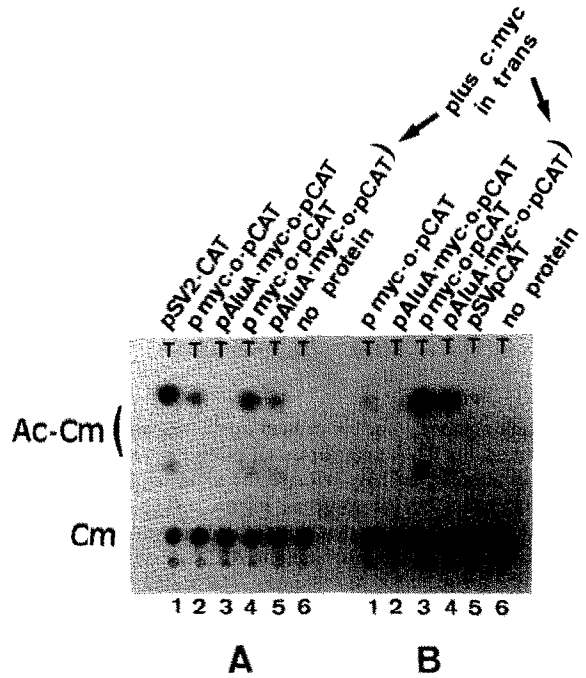

Fig. 2. Analysis of the effect of the Alu-derived sequence (AluA) on CAT expression driven by the *c-myc* enhancer (MRE) and SV40 promoter. 10 μg of the respective plasmid was introduced into HeLa cells (100-mm plates, 30–50% confluency, maintained in DMEM medium supplemented with 4% fetal calf serum and 6% bovine serum) by the Ca-phosphate method with 1 min glycerol shock. After 2 days, cells were collected without trypsin, lysed by sonication, and CAT assays were done as described earlier [9]. (A) and (B) two independent transfection experiments. The plasmid pSV2CAT contains both an enhancer and promoter of SV40; pSVpCAT contains only a promoter.

dent transfection experiments with different carrier plasmid DNAs, suggesting that the transcription silencer is present within the 13 bp AluA sequence (GGAGGCTGAGGCA).

Competition band-shift assays indicate that HeLa nuclear extracts contain a protein interacting with the AluA sequence [4], but in this study, we checked this by a direct band-shift assay using non-cloned AluA oligonucleotide 5′ end-labeled with ^{32}P (Fig. 3). The formation of protein-AluA complexes was not inhibited even in the presence (9th lane) of 100 ng of linearized pUC18 (2 μg of poly (dI-dC) was also included in all variants) but suppressed by the same amount of salmon sperm DNA (3rd lane), which is known as a strong inhibitor of the binding of human protein to the full-length BLUR8 Alu fragment. The results of the band-shift assay suggest that the AluA sequence interacts with HeLa nuclear protein. It seems possible that this interaction is responsible for the transcription silencing effect of AluA (Fig. 2), since a homologous GGAGGC-containing sequence (AluB, see [4]), also interacting with the HeLa protein, is situated at the left end of Alu repeats within the bipartite promoter for RNA polymerase III, just between enhancing and directing elements of the promoter [4].

We have also studied the influence of the AluA sequence on the ARS activity of MRE [9] using a transfection assay. The appearance of a DpnI-resistant plasmid DNA was analyzed by transformation of *E. coli* cells to ampicillin resistance (Table I). The pair of AluA-free and AluA-containing MRE plasmids without the SV40 promoter-CAT transcription unit was used (Table I, lines 1 and 2) along with the pAluA-myc-o-pCAT/p myc-o-pCAT pair (Table I, lines 3 and 4). The results (Table I) indicate that AluA suppresses ARS activity of MRE which was apparent when pSRalpha-myc was used as a carrier DNA (lines 1–4). DpnI-resistant plasmid DNA appeared after transfection of HeLa cells with the CAT-free pair of plasmids using Southern hybridization, and, again, AluA suppressed replication driven by the MRE ARS element (data not shown). It seems, therefore, that the Alu repeat-derived sequence (AluA) acts as a silencer in both transcription and initiation of DNA replication.

The results are not very surprising, if we assume that Alu retrotransposition [13,14] is regulated at the level of Alu transcription. The human haploid genome contains nearly a million copies of this mobile gene [12] and further multiplication of Alu might be deleterious because of insertion mutagenesis. Human cells possibly

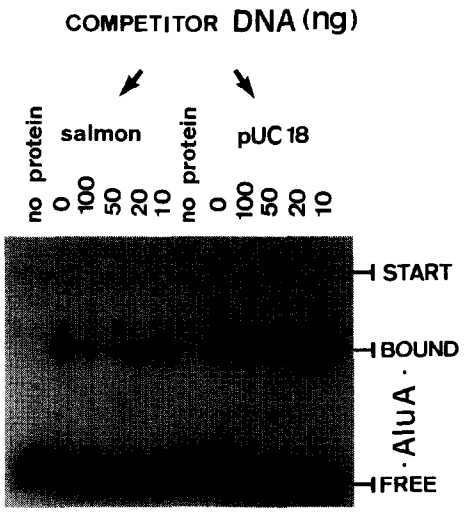

Fig. 3. Mobility shift assay of the binding of non-cloned AluA oligonucleotide by HeLa nuclear protein in the presence of the indicated amounts of salmon sperm DNA or of linearized pUC18. 2 μg of poly(dI-dC) was also included in all variants, as well as 10 μg of protein of HeLa nuclear extract. AluA2 oligonucleotide [4] was 5′ end-labeled with ^{32}P using phage T4 polynucleotide kinase, isolated on a column of Sephadex G25 and then annealed to a 2-fold molar excess of non-radioactive AluA1 to form AluA. 5 ng of AluA was incubated [4,5] for 10 min at room temperature with protein and run in a 4% polyacrylamide gel (19:1 crosslinks) in 0.25 × TBE buffer.

Table I

The influence of the Alu-derived sequence (AluA) on the MRE-driven replication of plasmid DNA in HeLa cells

Tested DNA	Carrier DNA	Number of plasmid clones rescued into *E. coli* after treatment of re-extracted DNA with DpnI
p myc-o	pSRalpha-myc	21
p AluA-myc-o	pSRalpha-myc	1
p myc-o-pCAT	pSRalpha-myc	102
p AluA-myc-o-pCAT	pSRalpha-myc	2
pUC18	no	3

Methods: 10 μg of tested plasmid and 10 μg of carrier DNA (or 20 μg of pUC18, last line) was introduced by the Ca-phosphate method into HeLa cells and after 3 days, cells were lysed and the low molecular weight DNA fraction (Hirt supernatant) was isolated as described [20]. After phenol and chloroform extraction, the DNA was washed by repeated precipitations with ethanol at room temperature, and treated with nuclease DpnI (Boehringer), which degrades non-replicated GATC-methylated input DNA, and introduced into competent (Ca method) *E. coli* C600 cells with selection of ampicillin-resistant clones. The efficiency of transformation of *E. coli* was 10 million of ampicillin-resistant clones per μg of pUC18. The efficiency of DpnI cleavage of GATC-methylated DNA under the conditions used for treatment of Hirt-supernatant DNAs was 99.8% (checked with non-transfected pUC18). 10 randomly chosen clones in the variant shown in line 3 were analyzed by the mini-prep method

developed a mechanism of repression of Alu transcription and transposition via a special repressor protein, but the question arises why Alu could not avoid this repression by a mutational drift? The answer is unknown, but the GGAGGC sequence might be important not only for repression of Alu transcription but also for other steps of transposition, e.g. for reintegration of reverse transcripts, which could explain conservation of the sequence [4,8]. Negative control of Alu transcription should allow modulation of the rate of insertional mutagenesis, and, maybe, of expression of structural genes, since many Alu repeats are present within introns and other non-coding regions of structural genes and an Alu silencer should influence their expression, if the silencer action could be distant. Interestingly, GC-rich silencer elements are found in promoters of some genes [21,22] and are suggested to play an important role in the control of gene expression [22].

The Alu-derived conserved GGAGGC sequence (AluA) also influences the initiation of DNA replication from the MRE ARS element in transfection experiments (Table I), and it is known that the region located 2.5–3.5 kb upstream of the non-rearranged human *c-myc* gene (which covers MRE) could serve as a chromosomal ORIGIN of DNA replication in vivo [15]. The possible presence of Alu repeats near chromosomal ORIGINs, therefore, could influence their activation, if Alu replication silencer action could be distant. An excessive number of Alu repeats in some chromosomal bands [16,17] might reflect a tendency to compensate for a high number of potential ORIGINs possibly present in these bands. Alu binding protein and SV40 T-antigen seem to have similar binding sequences [3–5,18], and it is known that SV40 T-antigen stimulates initiation of replication of chromosomal DNA in vivo [19]. This could be explained by a partial suppression of Alu silencers because of interference of T-antigen and Alu binding protein, which leads to activation of cryptic replication ORIGINs.

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