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Two Internal Sequence Elements Modulate Transcription From the External Human 7S K RNA Gene Promoter In Vivo

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Constructs of the external promoter of the human 7S K RNA gene in combination with different reporter elements and varying amounts of 7S K internal sequences were analyzed for efficient transcription by RNA polymerase III (pol III) in vitro and in vivo. In vitro, the 7S K promoter alone (−245 to −1) revealed full activity, compared to the entire wild-type gene. In vivo, however, the activity of the gene-external 7S K promoter, albeit clearly functional by itself, was positively modulated by internal sequence elements. Fusion constructs containing increasing amounts of transcribed 7S K sequences revealed that two elements were responsible for this activation. One element is associated with the initiator region (+1 to +8) of this class III gene. The second sequence comprises the 5′ half of a cryptic A-box starting at +10 of the 7S K RNA sequence. In the context of a totally unrelated vector sequence, a GGC element alone was sufficient to functionally replace that cryptic A-box. Thus, it appears that in context of the 7S K RNA gene—and possibly the 7S L and U6 RNA genes as well—structurally divergent A-box-like elements function as internal modulators of these pol III promoters.

7S K promoter Internal elements Polymerase III External promoter

TRANSCRIPTION of class III genes (genes transcribed by RNA polymerase III) has been studied extensively by analyzing promoter structures, identity of control elements, and the involvement of general and gene-specific transcription factors [reviewed in (9,29)]. According to their respective promoter structure, class III genes have been grouped into four different types (30). 5S RNA genes represent members of the first group with transcription being initiated by binding of the sequence-specific transcription factors TFIIIA and TFIIIC to the internal control region (ICR), followed by recruitment of the general factor TFIIIB as well as RNA polymerase III. The second group of entirely gene-internal promoters is found in tRNA and the adenoviral VA RNA genes. Here binding of TFIIIC to the A- and B-box elements is followed by positioning of TFIIIB upstream of the transcription start site and subsequent recruitment of the pol III enzyme. The third group, represented by the split promoters of the 7S L RNA and the viral EBER RNA genes, is characterized by gene-internal A- and B-box sequence elements supplemented with external promoter motifs, such as ATF and Sp1 binding sites located about 40–60 bp upstream of the transcription start site. Transcription from the fourth group of pol III promoters, found in 7S K RNA and U6 RNA genes, depends on three exclusively gene-external sequence elements that are also found in genes of the RNA polymerase II transcription system: a canonical TATA-box around −25, a proximal sequence element (PSE) at −50, and a distal sequence element (DSE) located around −240 [(32), and references therein]. As summarized for the human U6 (10) and 7S K (1) RNA genes, the functional significance for transcriptional regulation of each of these external promoter elements was verified. Therefore, in general three sequence elements are necessary and sufficient for transcription initiation from those group IV promoters in vivo and in vitro. Yet, with the exception in case of the human 7S K promoter, the DSE was found

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dispensable for transcription in vitro but not in vivo (14).

Up to now, however, no studies have been performed to investigate a possible modulation of the basal activity of these external group IV promoters by gene-internal sequences. Here, we present a detailed analysis in vivo and in vitro of 7S K promoter constructs containing varying amounts of gene-internal sequences. These experiments identified two internal sequence elements modulating the activity of the external promoter of the human 7S K RNA gene.

MATERIALS AND METHODS

Templates

The basic human 7S K(−3)/pAT fusion construct used for expression studies in vivo has been described in detail previously (13). The corresponding constructs 7S K+8/pAT, 7S K+30/pAT, 7S K+8/pAT plus 5′-GGC, and 7S K+8/pAT plus 3′-GGC were obtained by insertion of synthetic oligonucleotides between the Asp718 site of the 7S K RNA gene (−7) and in the *Cla*I site of the pAT153 vector sequence (+24). Hence, the transcribed sequence of these constructs had the same length.

A similar VA reporter gene construct contained the adenoviral VA I RNA sequence from +72 to +140, supplemented with the 7S K termination region (+269 to +331) (8). This reporter cassette was fused with its *Xba*I site either to the Asp718 site (−7), to the *Sau*3AI site (+13), or to the *Hin*fI site at +55 (5′ overhangs filled in with Klenow enzyme) to obtain the constructs 7S K−2/VA, 7S K+16/VA, and 7S K+58/VA, respectively. All other VA reporter constructs were cloned by insertion of synthetic oligonucleotides between the Asp718 site of the 7S K RNA gene and the *Xba*I site of the 7S K+58/VA construct (with the *Xba*I site being restored).

The EBER-2 RNA gene (−156 to +170) (24) used in some experiments for normalization of transfections was amplified by PCR from a cosmid containing the 30-kb cM *Sal*A fragment of the Epstein-Barr virus. That fragment was inserted into the *Bam*HI site of the KS+ vector.

In Vitro Transcription

Nuclar extracts (NE; 6–8 mg/ml of protein) were prepared from HeLa cells as described (7). In vitro transcription assays with 10 µl of extract and 1 µg of plasmid DNA were as described previously (13) in a total volume of 50 µl in the presence of 0.5 µg/ml of α-amanitin. Reactions were terminated by the addition of lauroylsarcosin (1%) and phenol-extracted transcripts analyzed in 6% polyacrylamide gels containing 8 M urea, followed by PhosphoImager (Fuji, Tina2.09) quantification or autoradiography. Exposure to Fuji X-ray films was for 12–16 h using a Cronex intensifier screen.

Transfection and S1 Nuclease Analysis

7S K RNA and EBER-2 RNA constructs were transfected into HeLa monolayer cells by the DNA-calciumphosphate-coprecipitation method (11) using about 20 µg of superhelical plasmid DNA per 9-cm plate. After incubation for 16 h at 37°C in the presence of 3% CO_2, the cells were washed with DMEM and incubated with fresh medium for another 24 h in the presence of 7% CO_2. For extraction of RNA, cells were lysed on plates by the addition of 4 M guanidinium thiocyanate in the presence of 0.5% lauroylsarcosin, followed by extraction with phenol/chloroform. and ethanol precipitation. S1 nuclease protection analyses of cellular RNA after hybridization with labeled DNA fragments were as described previously (8). In case of the 7S K/VA constructs, probes were 3′ end-labeled by filling in with Klenow enzyme of an *Xba*I restriction site at the 5′ end of the VA cassette, resulting in a protected fragment of 158 nt in length. Similarly, corresponding transcripts from 7S K/pAT constructs were detected as a protected band of 215 nt with DNA 3′ end-labeled at a *Cla*I site at position 23 of the pAT153 vector sequence. The analysis of EBER-2 reference transcripts was with probes, 3′ end-labeled at the *Ava*I site at +75 of the wt gene, and showed a protected fragment of 95 nt in length.

RESULTS

Gene-Internal Sequences Near the Transcription Start Site Activate the 7S K Promoter In Vivo But Not In Vitro

In accordance with the U6 snRNA gene (6,16), transcription of the 7S K RNA gene is clearly under control of a gene-external promoter in vitro and in vivo (13,19). However, our analyses of 7S K promoter/reporter (−1/+1) fusion constructs reproducibly revealed comparatively low activity in vivo. In contrast, in vitro these constructs showed wild-type level transcriptional actvities. Therefore, we wanted to know whether or not this difference might be attributable to the presence of gene-internal sequence elements. For this, two types of promoter fusion constructs were cloned using either a procaryotic vector sequence of the pAT153 plasmid as reporter or an adenoviral (pol III transcribed) VA I RNA element.

These sequences were fused either to position −3 or +8 of the 7S K RNA promoter. As is evident from the in vitro transcription analysis shown in Fig. 1A, with both reporter constructs no difference could be observed between the −3 and +8 fusions, indicating that sequences around the transcription start site had no influence (compare lanes 1 and 2 and lanes 3 and 4, respectively). The lower band in all four lanes, representing a newly identified U6 snRNA-specific terminal uridylyl transferase activity present in these extracts (26), can be taken as normalization control for these assays. Furthermore, the identity of the reporter element also appeared to have no influence on transcription in vitro. In contrast, a very different picture emerged from the analysis of the same constructs in vivo. The comparison of lanes 1 and 2 of Fig. 1B reveals that the fusion of the pAT reporter sequence either to position −3 or to +8 of the promoter had a dramatic effect on transcription of these constructs in vivo. While in these S1 nuclease analyses a clear signal was obtained with transcripts of the +8 fusion construct (lane 2), protected fragments were hardly detectable with RNA transcribed from the corresponding −3 fusion (lane 1). The same holds true for the results obtained with the analogous VA reporter constructs (lanes 3 and 4). Again, a clear signal was observed with the +8 fusion construct, which by intensity came close to the cotransfected EBER-2 normalization control (see "ref" in lanes 1–4). On the other hand, only an extremely weak band was obtained again with RNA from the −3 clone. Together, these results indicate that transcription from these promoter constructs in vivo, but not in vitro, is influenced by sequence elements located around the transcription start site (+1).

Compared to the wt sequence, the initiator region of the −2/pAT fusion construct (with the pAT reporter sequence starting at −2) differed by two aspects (see sequences in Fig. 2A): the two pyrimidine nucleotides (−2/−1) immediately upstream of the start site were deleted. As shown previously (13), in that construct initiation of transcription in vitro was relocated and occurred at the first "G" of the GAGCTC sequence. Secondly, the sequence of the three 5′ purines was converted from GGA to GAG. To see whether one of these changes might be responsible for the inactivation observed, three additional clones were analyzed in comparison to a 7S K(+58)/VA

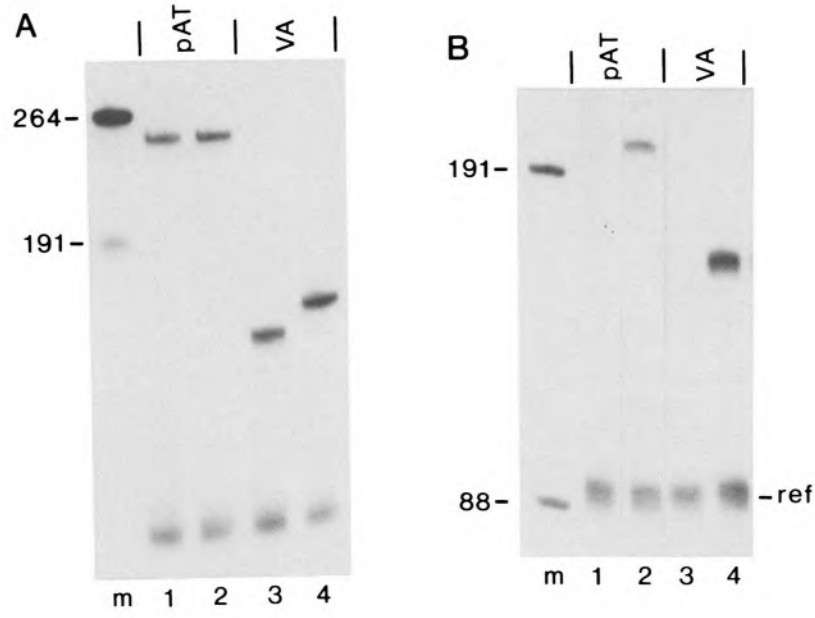

FIG. 1. Transcription from 7S K promoter constructs with and without internal sequences in vitro and in vivo. (A) In vitro transcription in S 100 extracts of 7S K promoter constructs with either a procaryoptic vector sequence (pAT; lanes 1 and 2) or an adenoviral VA I fragment (VA; lanes 3 and 4) as reporter. Fusion of the reporter cassette was either to position −3 (lanes 1 and 3) or +8 (lanes 2 and 4) of the 7S K RNA gene. Phenol-extracted, labeled transcripts were analyzed directly in denaturing 6% polyacrylamide gels. The lower band in lanes 1–4 is the product of a newly described U6 snRNA-specific terminal uridylyl transferase (26) and may be used for normalization. (B) Expression in vivo of the same constructs as in (A). Plasmid DNA (18 μg) was transfected into HeLa cells in the presence of 2 μg of a plasmid containing the EBER-2 RNA gene, as normalization control ("ref"). In this case, however, analysis of transcripts was indirectly by S1 nuclease protection of labeled DNA fragments (see Materials and Methods). Protected fragments were 215 nt for the pAT construct (lanes 1 and 2), 158 nt for the VA cassette (lanes 3 and 4), or 95 nt for the EBER-2 reference, respectively. m = marker DNA fragments.

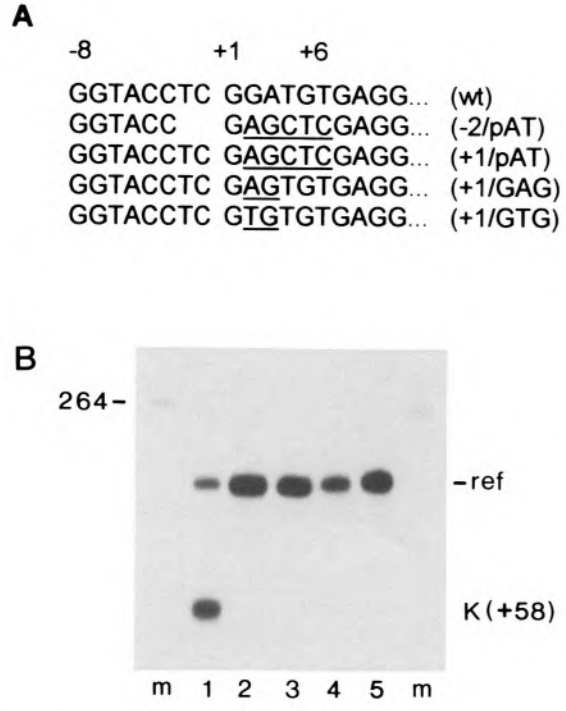

FIG. 2. Analysis in vivo of mutants with altered initiator sequence. (A) Initiator sequences (region −8 to +10) of the constructs analyzed in comparison to the wild-type clone. The constructs analyzed were based on a wild-type promoter construct with the VA reporter cassette fused to position +58 of the 7S K RNA gene. Internal mutations (underlined) and the deletion of two upstream base pairs are indicated and were obtained by insertion of synthetic oligonucleotides with emphasis on a G nucleotide being preserved at position +1. (B) S1 nuclease protection analysis after transient expression of the constructs in A (10 μg), together with 20 μg of construct 7SK(+8)/pAT of Fig. 1, as reference (ref). Lane 1 = wt (K+58); lane 2 = −2/pAT; lane 3 = +1/pAT; lane 4 = +1/GAG; lane 5 = +1/GTG; m = marker.

construct, here regarded as wild-type (wt) because it contained the entire external promoter, the authentic initiatior region, and the first 58 bp of the transcribed 7S K RNA sequence. In the first new construct, the missing −2/−1 bp were introduced again, with the pAT reporter sequence now beginning at +1 (+1/pAT). The other two constructs contained double-point mutations, converting the initial trinucleotide (GGA) of the wt construct into "GAG" or "GTG" (+1/GAG and +1/GTG), respectively. After transfection and S1 nuclease protection analysis, only the wt K(+58) construct was found active (Fig. 2B, lane 1), whereas no transcripts of the other clones were detectable (lanes 2–5). Together, these results allowed two conclusions: one is that omission of the −2/−1 bp was not responsible for the inactivation observed before, and, secondly, the sequence downstream of the transcription start site is important for efficient expression of the gene.

The First Eight Base Pairs of the 7S K RNA Gene Are Required for Efficient Expression In Vivo

In our earlier studies on the human 7S K RNA gene we had seen that the +1 G nucleotide of the gene is essential for efficient initiation of transcription. The results presented in Fig. 2 have extended this significance to base pairs +2/+3. In order to determine the extension of the entire initiator region, a series of 7S K/VA constructs was cloned with increasing amounts of 7S K sequence being present, as indicated by the numbers above each lane of Fig. 3. As before, these constructs were analyzed either by in vitro transcription (Fig. 3A) or after transfection in intact cells (Fig. 3B). The results summarized in Fig. 3C were obtained by PhosphoImager quantification of the signals of four independent experiments as in Fig. 3A and B and after normalization for internal controls, as in Fig. 1. These data reveal that efficient expression in vivo of these 7S K promoter/reporter constructs depended on the amount of gene-internal sequences present. Starting with the −2 fusion, a steady increase in activity was observed up to the +8 construct, which is slightly (about 10%) less active than the +30 clone (set as 100%, because the presence of additional 7S K sequences up to +58 did not further increase activity; data not shown). In contrast, in vitro analyses of the same constructs did not reveal a comparable dependence in transcription efficiency on the presence of increasing amounts of internal sequences, although some variation around the 100% level of the +30 clone was observed with different constructs. It should be noted that due to the way of construction (see Materials and Methods), direct analysis of RNA synthesized in vitro (Fig. 3A) in some cases must result in different lengths of transcripts, whereas the S1 nuclease protection analysis in Fig. 3B in all cases aimed at the same reporter element. Together, these results indicate that in vivo, but not in vitro, the external human 7S K promoter is modulated by an internal initiator sequence element that extends at most 8 bp downstream of the transcription start site.

From the comparison of the +8 and +16 constructs with the full +30 activity it appeared, however, that a second, albeit minor, element located further downstream might also contribute to maximal expression in vivo. This assumption was also supported by the earlier data in Fig. 1B which showed that different reporter sequence elements (pAT or VA), both fused to the +8 position of the 7S K gene, resulted in different activities of the two constructs in vivo. The analysis of the 7S K RNA sequence between positions +8 and +30 revealed a cryptic A-box element (5′-GGC GATCTGG-3′) with 5′ and 3′ ends (but not the cen-

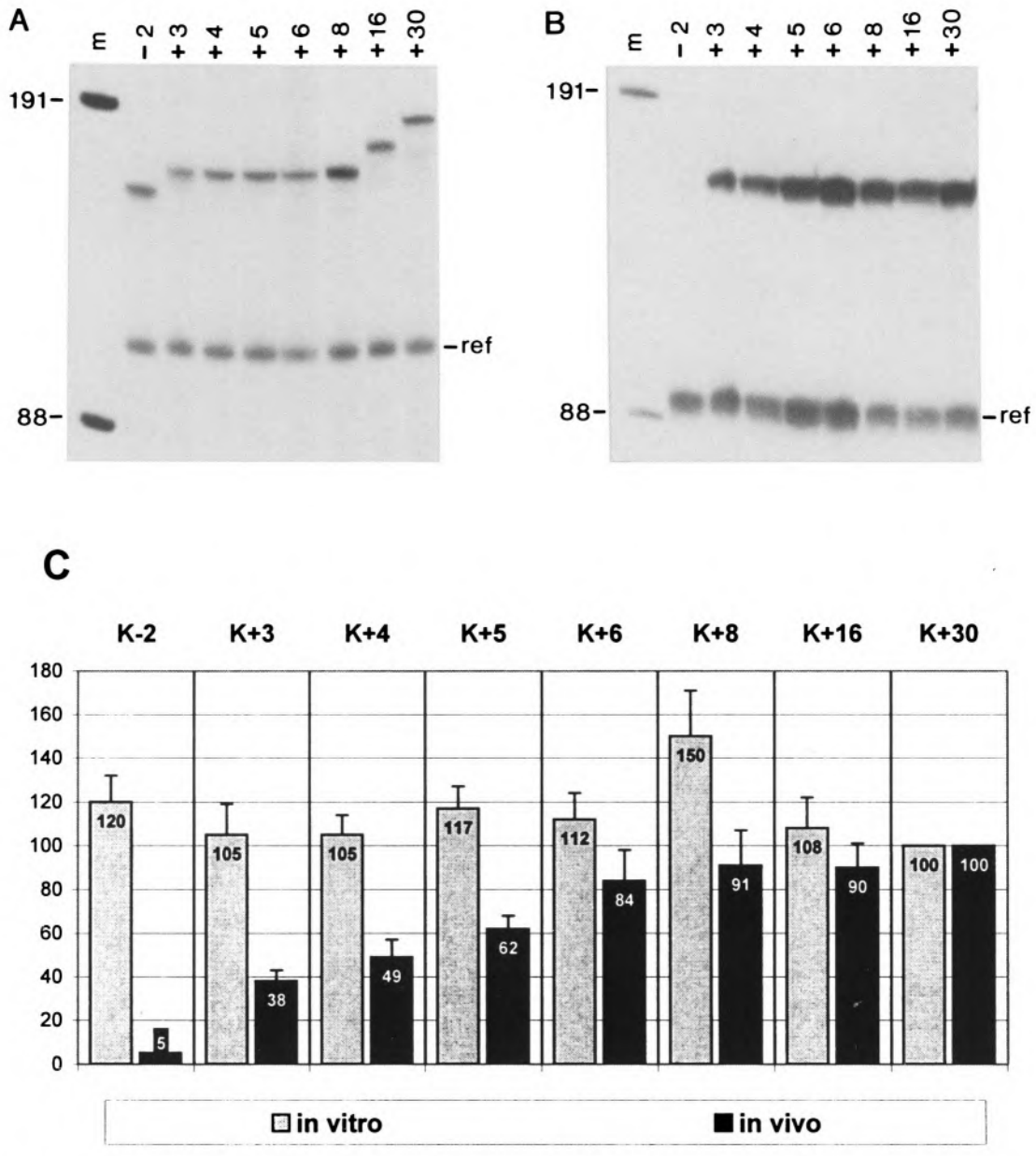

FIG. 3. Analysis of initiator region mutants containing deletions downstream of the transcription start site. Seven VA reporter constructs (numbering +3 to + 30 refers to the last position of the 7S K RNA gene sequence retained) were analyzed in vitro and in vivo in comparison to the −2/pAT construct of Fig. 2. (A) In vitro transcription reaction products analyzed as in Fig. 1A, with the lower reference band representing in vitro-labeled U6 snRNA, suitable for normalization. The different lengths of transcripts are due to the construction of clones (see Materials and Methods). (B) In vivo expression after transfection of the same constructs as in (A). The lower band (ref) represents the normalization control obtained with a cotransfected EBER-2 RNA plasmid. Because in this case S1 nuclease protection was based on the VA reporter sequence only, all bands reveal the same protected fragment. (C) Graphic presentation of a PhosphoImager quantification of four independent experiments performed with these constructs in vitro and in vivo. In all cases, individual signals were normalized for their respective references.

ter) matching the consensus sequence (5′-GGCN NARYGG-3′) as defined originally (4). To analyze the significance of such a limited A-box homology in more detail, we compared pAT and VA reporter fusion constructs (Fig. 4) directly. For this 7S K promoter constructs were cloned with the pAT (Fig. 4A) or VA (Fig. 4B) reporter sequence starting at +1, +8, or +30 of the K gene. In both cases, the analysis in duplicates of these constructs in vivo confirmed the significance of the initiator element studied before. However, in contrast to the K+8/VA construct, which as before revealed about 90% activity compared to

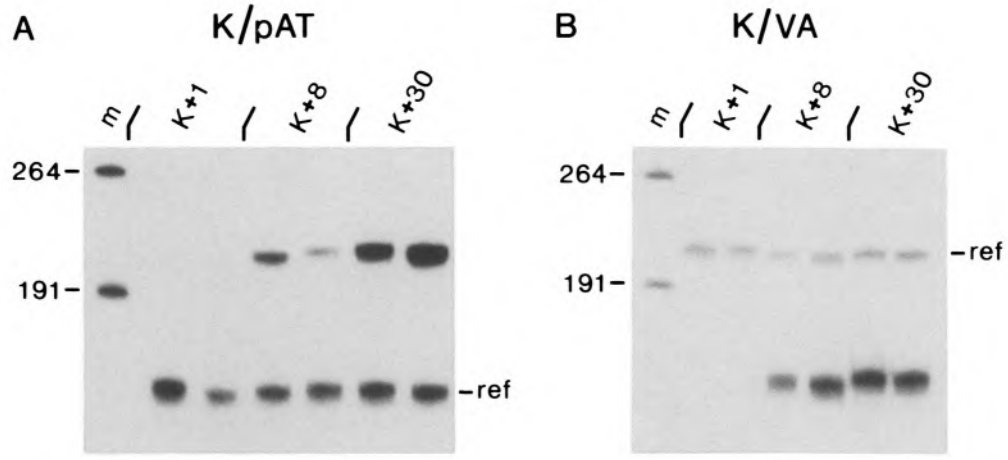

FIG. 4. Sequences downstream of position +8 modulate transcription from the 7S K promoter in vivo. Expression rates of constructs with either the pAT sequence or the VA cassette as reporter and in combination with increasing amounts of the 7S K internal sequence were compared in duplicate assays. Numbering +1 to +30 indicates where the reporter sequence started. (A) 7S K–pAT fusion constructs were analyzed in comparison to the K+58/VA reference. (B) Expression of the corresponding 7S K–VA fusions with the K+8/pAT construct as reference.

the corresponding +30 construct, the corresponding K+8/pAT construct showed only about 40% of activity, compared to the analogous +30 construct (Fig. 4B). It should be noted that in these analyses the activity of the K/pAT constructs was normalized for the activity of a cotransfected K+30/VA reference and, vice versa, the activity of the K/VA constructs for K+8/pAT, as reference. Together, these results indicate that sequences located downstream of the initiator element are also able to modulate transcription of the 7S K promoter/initiator construct in vivo.

"GGC" Elements Are Able to Replace the Cryptic A-Box Functionally

The results in Fig. 4 had shown that the activity of the K+8/VA construct was twice as much, compared to the corresponding K+8/pAT construct. Yet, the sequence analysis of the respective VA reporter element did not reveal any significant homology to an A-box, neither to the consensus nor to the cryptic A-box present in the +30 clones. At best, the 5′ half of an A-box (5′-GGCNN-3′) could be found at position +18 to +22 of the K+8/VA construct (not shown). Our studies on internal elements of the human 7S L RNA gene had identified a similar 5′ half of an A-box as a functional modulator of that promoter (Krüger and Benecke, unpublished). Therefore, we focused our attention on the two GGC elements associated with the 7S K wt sequence at positions +10/+12 and +18/+20, respectively (see sequences in Fig. 5A). Based on the K+30/VA clone, a construct was generated lacking both these GGC elements (minus 5′ and 3′ GGC). This clone was analyzed in comparison with a similar construct from which only the 5′-GGC element at position +10/+12 had been deleted (minus 5′-GGC) and with the previously used K+16/VA construct, which in this context can be regarded as a clone lacking the 3′-GGC element at position +18/+20 (minus 3′-GGC). In comparison to the fully active K+30/VA construct (100%), all these "minus GGC" constructs showed clearly reduced transcription rates in vivo (relative values obtained after PhosphoImager quantification and normalization for the reference are given in brackets behind the sequences). The analysis performed with these mutant clones in vivo (see bands in Fig. 5A) reveals that deletion of both GGC elements resulted in a two thirds loss in activity, down to 35% of the K+30/VA clone. The presence of either of the two GGC elements alone mediated a significant rescue in activity (90% and 74%), yet without reaching full activity (K+30/VA) again. Furthermore, from the comparison of the "minus 3′-GGC" and "minus 5′-GGC" constructs it might appear that the GGC element closer to the transcription start site was slightly more efficient than the element located further downstream.

In order to demonstrate more directly the significance of the GGC elements identified above, we studied the effect of these elements in the context of a completely unrelated sequence. For this, the basic 7SK/pAT construct (K+8/pAT, containing the entire

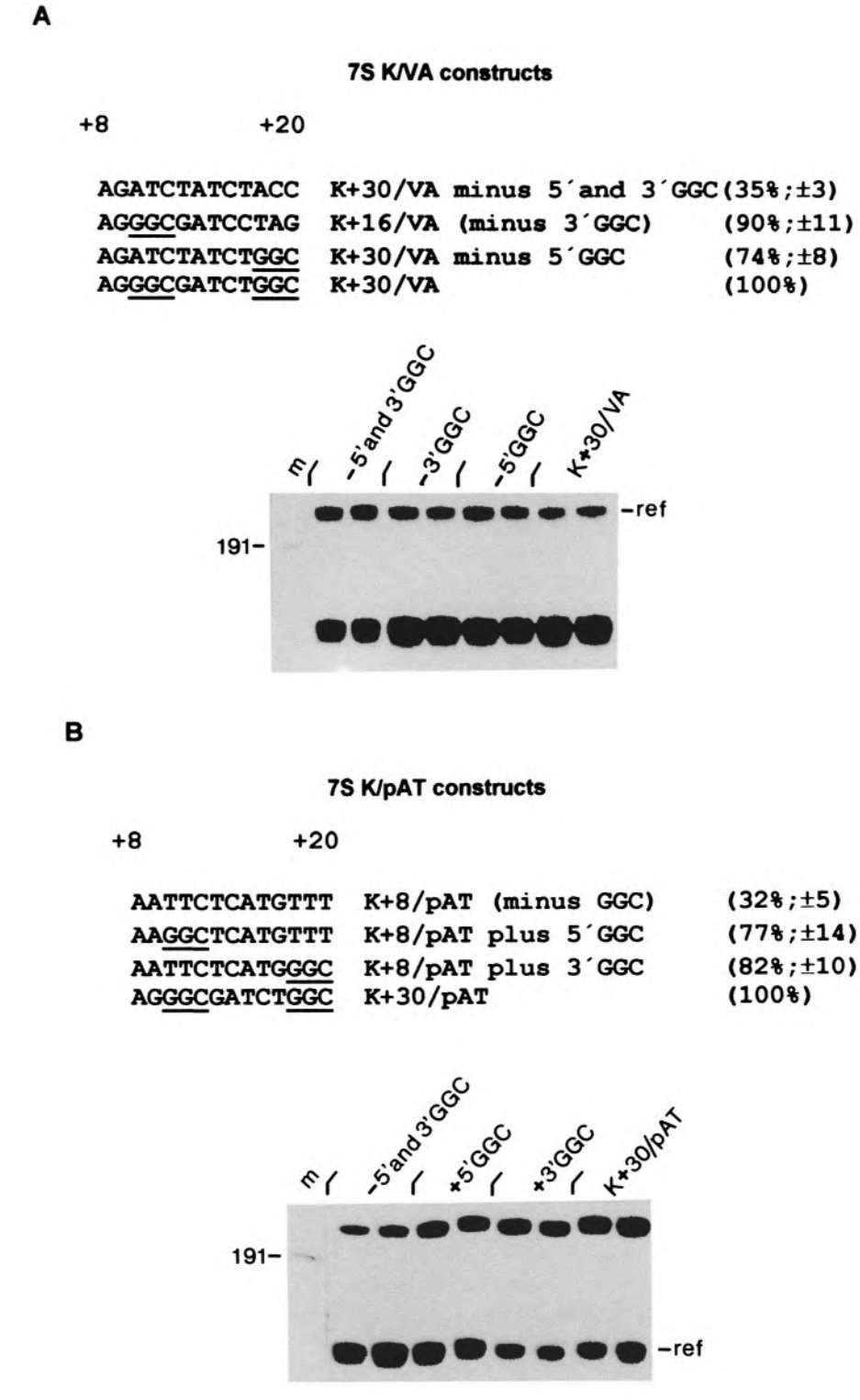

FIG. 5. The significance of internal "GGC" sequence elements for the expression of 7S K reporter constructs. (A) 7S K/VA constructs containing both GGC elements (K+30/VA), only the 5′-"GGC" (K+16/VA), only the 3′-"GGC" (K+30/VA minus 5′-GGC), or none of the two GGC elements (K+30/VA minus 5′- and 3′-GGC) were analyzed in comparison to the 7S K+8/pAT reference. (B) Similar constructs as in (A) but with the pAT sequence as reporter were analyzed in comparison to the 7S K+58/VA clone, as reference. In both parts, relative expression rates as obtained in four independent experiments are indicated in the brackets in percent, supplemented with standard deviations. As before, PhosphoImager quantification of the signals included normalization for the respective reference bands. For clarification, individual sequences downstream of the initiator region (from +8 to +20) are listed on the top of each section.

gene-external promoter and the authentic initiator region preceeding the procaryotic vector sequence) was supplemented with either of the two GGC elements by insertion into the reporter sequence and then analyzed again in vivo, in comparison to the K+30/pAT construct. As is evident from the results shown in Fig. 5B, again both the 5′- and the 3′-GGC elements by themselves were able to stimulate transcription more than twofold (to 77% and 82%, respectively), compared to the activity of the "minus GGC" K+8/pAT construct (32%). Again (see Fig. 5A), however, constructs with only one GGC element fell short of the full activity of the K+30/pAT clone. Together, these results indicate that in addition to the positive effect mediated by the eight base pairs of initiator region, the presence of a second modulatory element is required for full activation of the external promoter of the human 7S K RNA gene in vivo.

DISCUSSION

Among the four different classes of pol III promoters, those controlling the 7S K and U6 RNA genes most closely resemble the promoters of protein-encoding genes transcribed by RNA polymerase II. In general, in pol II genes a core promoter consisting of several *cis*-acting elements is modulated by upstream enhancer sequences [reviewed in (32)]. In case of the pol III-transcribed 7S K RNA and U6 snRNA genes, the core promoter is composed of the TATA-box and the highly conserved proximal sequence element (PSE) (10,17,18), also found in the RNA polymerase II-transcribed U-snRNA genes (5,17,31). The distal sequence element (DSE) of the U6 RNA gene is represented by an octamer factor binding site (2) while function of the 7S K DSE depends on a CACCC element (14). Therefore, together with a functional TATA-box, these two pol III genes are controlled by proximal as well as distal promoter elements that previously had been identified in the pol II transcription system.

In addition to their well-characterized promoter structure, essential sequence elements have been identified around the transcription start site of pol II genes (25). By sequence homology, these initiator elements (Inr) can be grouped into different families (3). The functional analysis of several Inr elements has significantly contributed to our understanding of transcriptional regulation of protein-encoding genes, with particular emphasis on the mechanistic differences in initiation complex formation observed between TATA-containing and TATA-less promoters [reviewed in (28)].

Furthermore, a detailed analysis of the 5′ end of the *Acanthamoeba castellanii* ribosomal RNA transcription unit revealed a sequence element with significant homology to the initiation sequences of a variety of rRNA genes from other species (21). These results indicated that in the RNA polymerase I system too, transcription initiation might depend on the presence of an efficent initiator element. Interestingly, this Inr alone was able to drive weak transcription in the presence of only one factor (TIF-IB) and the RNA polymerase itself (22).

Our results on the significance for expression in vivo of sequences immediately downstream from the transcription start site of the human 7S K RNA gene extend the analogy described above between pol I and pol II promoters and the group IV gene-external promoters of the pol III transcription system. Up to now, initiator sequences important for initiation by RNA polymerase III have only been thought of as "purine-rich" elements at the transcription start site, preceeded by pyrimidine nucleotides. Our analysis of the 7S K initiator element demonstrate, however, that the mere presence of purines at the transcription start site is not sufficient. Rearrangement of the first three purines of the wild-type GGA sequence to GAG (see construct +1/GAG in Fig. 2) resulted in a complete loss of activity in vivo. This result, together with the finding that more sequences than just the first three purine nucleotides of the wild-type sequence are essential for efficient initiation, indicates that a real initiator element does exist within this pol III-transcribed sequence of the human 7S K RNA gene as well. In the pol II sytem, protein factors specifically binding to the initiator element have been identified recently. In particular the Ying-Yang 1 protein (YY1) has attracted considerable interest, because this factor provides the only example of eukaryotic transcription initiation in the absence of the TATA-binding protein TBP (27). In general, TBP is known to play a central role in transcription by all three eukaryotic RNA polymerases (12,23). In a preliminary attempt, we have been looking for nuclear or cytoplasmic extract proteins specifically recognizing the 7S K initiator sequence element. Using band-shift assays, we were not yet able to detect specific binding of a cellular protein to this sequence. It is quite conceivable, however, that such an interaction will be detectable only if at least partially purified extract fractions are applied, as seen for example in case of the YY1 protein (27).

The second internal sequence element modulating transcription from the 7S K promoter seems to be related to the wide-spread A-box elements of various pol III genes. These A-box elements represent the binding sites for the well-characterized eukaryotic transcription factor TFIIIC (29). Two important fea-

tures seem to discriminate the A-box-"like" element found in the 7S K wt sequence from those authentic A-boxes present as essential elements within the internal promoters of, for example, tRNA and adenoviral VA RNA genes. First, the A-box element found in the 7S K RNA sequence shows only a limited homology to the A-box consensus (4). It clearly lacks the center of that box with the essential A nucleotide. Second, a 7S K promoter construct that contains the entire upstream sequence plus the initiator element (Inr) but no elements downstream of position +8 (see K+8/pAT in Figs. 4A and 5A) still is clearly active, representing more than 30% of the wild-type activity in vivo and full activity in vitro (Fig. 1A). Therefore, it appears that in contrast to the aforementioned A-box promoters of tRNA and VA RNA genes, the GGC element identified here is not essential for transcription initiation by RNA polymerase III, but rather represents a modulating downstream sequence element. These functional differences might point—at least in case of the 7S K RNA gene—to the existence of less conserved A-box-like elements. One might argue, however, that the GGC element described here is not related to the A-box element at all. In that case, one has to assume that this element is specifically recognized by another protein than the well-characterized general transcription factor TFIIIC (15). In this context it is interesting to note that a partially purified TFIIIC fraction is able to stimulate K+8/VA construct (in which the VA sequence provides a GGC element now located between +18 and +20) but not the K+8/pAT construct analyzed in Fig.5B, which corresponds to a minus GGC clone (data not shown). This finding is in agreement with the observation that clearly different TFIIIC factors do exist, the basic form of which ($TFIIIC_1$) is considered to be involved in transcription of all pol III genes (15,29). Furthermore, recently a variant U6-specific TFIIIC factor (designated TFIIIU) was described that is supposed to be involved in transcription intiation of those pol III genes that depend on an upstream promoter (20). Although that analysis has not yet been performed, it is possible that TFIIIU may be the factor recognizing such a truncated A-box-like element (GGCNN) described here. TFIIIU is not functional in the context of "classical" A-box genes, such as tRNA and VA RNA genes, which lack upstream promoter elements (20). There, $TFIIIC_1$ is required and, in addition to RNA polymerase III and TFIIIB, is sufficient to promote initiation. It is quite conceivable, however, that in the case of the more complex upstream pol III promoters, from the gene-internal side only a less efficient factor is required that in turn recognizes a degenerated A-box. In such promoters (7S K and U6 RNA genes), a sophisticated preinitiation maschinery is already recruited by the TATA-box binding complex, in conjunction with DSE and PSE binding factors. A similar situation seems to be present in the split (internal and external) 7S L RNA promoter. There, out of two putative A-boxes that overlap each other, again the truncated one, which represents a GGC element only, is functional (Krüger and Benecke, unpublished). Thus, it appears that in accordance with their different structure, the internal A-box-like elements of upstream pol III promoters are also functionally different from those found in tRNA and VA RNA genes.

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