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Transcription-mediated binding of peptide nucleic acid (PNA) to double-stranded DNA: sequence-specific suicide transcription

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ABSTRACT

Peptide nucleic acid (PNA) forms sequence-specific $(PNA)_2$/DNA triplexes with one strand of double-stranded DNA by strand invasion. When formed with the template strand of DNA such a $(PNA)_2$/DNA triplex can arrest transcription elongation *in vitro* and can thus act as an anti-gene agent. One of the major obstacles to applying PNA as an anti-gene agent *in vivo* is that PNA strand invasion occurs at a very slow rate under moderate salt conditions. In the present study we show that transcription can increase the rate of sequence-specific PNA binding dramatically. Such transcription-mediated PNA binding occurs three times as efficiently when the PNA target is situated on the non-template strand as compared with the template strand. Since transcription can mediate template strand-associated $(PNA)_2$/DNA complexes which arrest further elongation, the action of RNA polymerase results in repression of its own activity, i.e. suicide transcription. These findings are highly relevant for the possible future use of PNA as an anti-gene agent.

INTRODUCTION

Molecules which are capable of binding double-stranded DNA in a sequence-specific manner have received much attention as they might be developed into tools for modulating gene expression and, at a later stage, be exploited as gene-targeted pharmaceuticals. Strong candidates for such purposes are oligonucleotides and their analogs (1,2) and possibly also DNA binding proteins (3).

Peptide nucleic acid (PNA) is an oligonucleotide analog in which the (deoxy)ribose phosphate backbone has been replaced by a homomorphous, achiral and uncharged *N*-(2-aminoethyl)glycine polymer. Homopyrimidine PNA can bind to a complementary homopurine DNA target, leading to a very stable $(PNA)_2$/DNA triple helix (4–7). Such a complex is sufficiently stable to displace the non-target strand of double-stranded DNA (4,8–10) and when formed with the template strand downstream of a promoter it can arrest transcription elongation (11–13), i.e. PNA may act as an anti-gene agent. However, $(PNA)_2$/DNA triplex formation with double-stranded DNA is severely inhibited by the presence of moderate levels of cations (e.g. 50 mM Na^+) (8,9,14). In contrast, $(PNA)_2$/DNA triplex formation involving single-stranded DNA occurs independently of ionic strength (6,7). The rate limiting step in the formation of the strand invasion complex is believed to be local distortion of the DNA double helix, commonly referred to as DNA 'breathing' (4,10).

As the transcriptional elongation complex possesses a single-stranded region, termed the transcription 'bubble' (15) we reasoned that by transcribing a DNA fragment containing a homopurine target sequence in the presence of a complementary PNA the rate of $(PNA)_2$/dsDNA complex formation should be increased. In this study it is shown that transcriptional activity can indeed catalyze the formation of $(PNA)_2$/dsDNA strand invasion complexes under ionic conditions at which spontanous strand invasion is exceedingly slow. This transcription-mediated triplex formation occurred when the PNA target was situated on the template strand as well as on the non-template strand. The relevance of these findings for the possible future use of PNA as a gene-targeted drug is discussed.

MATERIALS AND METHODS

PNAs

The bis PNAs H-$(Lys)_n$-TTJTTJTTTT$(eg)_3$TTTTCTTCTT-Lys-NH_2 (n = 4 for PNA 575, n = 3 for PNA 655) were synthesized and characterized as described previously (16,17). J and eg denote the synthetic nucleobase pseudoisocytosine and ethylene glycol (8-amino-3,6-dioxaoctanoic acid) linker units respectively. The multiple Lys residues in the PNA increase its positive charge and thus the rate of (PNA_2)/dsDNA complex formation (Nielsen *et al.*, unpublished results). In most experiments PNA 655 was chosen instead of PNA 575 as the latter had a tendency to cause aggregation of the template DNA (not shown)

DNA constructs

The 460 bp DNA fragments used as templates for *in vitro* transcription were excised with *Pvu*II from two different pBluescript KS+ (Stratagene) derivatives, pT8C2KS and

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pA8G2KS. These plasmids carried single PNA targets, inserted into the *Pst*I site, on either of the two strands (13). Both strands of the DNA fragments were dephosphorylated with alkaline phosphatase and were 5′-radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (18).

In vitro transcription

In vitro transcription reactions were incubated at 37°C for up to 80 min and contained 5–40 ng template, 1–10 µM PNA, 10 mM DTT, 0.5 mM NTPs, 6 mM $MgCl_2$, 3 mM spermidine, 5 mM NaCl, 40 mM Tris–HCl, pH 7.5, 100 ng/µl BSA and 20 U T3 or T7 RNA polymerase (Gibco BRL) in a total volume of 10 µl. In case the transcripts were intended for subsequent analysis the reactions were supplemented with RNase inhibitor (1 U/µl).

Permanganate probing

The transcription reactions were diluted with 90 µl DTT-free transcription buffer before the templates were oxidized with 13.3 µl 20 mM $KMnO_4$ for 15 s and stopped by adding 75 µl 1 M β-mercaptoethanol, 1.5 M NaOAc, pH 9. In the kinetics experiments excess DTT was oxidized with Br_2 prior to $KMnO_4$ probing (5 µl 20 mM $KMnO_4$ for 15 s). When the $(PNA)_2$/dsDNA complexes were preformed in low salt solution (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) they were supplied with the appropriate amount of transcription buffer (including DTT) prior to $KMnO_4$ probing so that all samples were oxidized under identical conditions. The oxidized DNA was ethanol precipitated and cleaved in 100 µl 10% piperidine for 20 min at 90°C. The samples were lyophilized, resolved using 7% polyacrylamide–7 M urea sequencing gels and subjected to autoradiography.

RNA analysis by hybridization

Aliquots (1 µl) removed from the T7 *in vitro* transcription reactions were stopped in 19 µl TER (10 mM Tris, pH 6.8, 1 mM EDTA, 100 ng/µl yeast RNA) and the RNA polymerase was heat inactivated (70°C, 10 min). The template DNA was degraded by incubating for 30 min at 37°C after addition of 1 µl 0.2 M $MgCl_2$ and 10 U RNase-free DNase. The RNA samples were diluted with 80 µl 20× SSC (1.5 M NaCl, 0.6 M sodium citrate) and vacuum blotted onto nylon filters (Hybond N+; Amersham) together with known amounts of RNA transcribed from T8C2KS/*Pvu*II. After UV fixation the filters were prehybridized for 3 h and hybridized overnight at 65°C in 0.25 M Na_2HPO_4, pH 7.6, 7% SDS, 1 mM EDTA. The hybridization probe was an [α-^{32}P]dATP random primer-labeled (18) 2.8 kb *Eco*RI–*Hin*dIII DNA fragment from pBluescript KS+ which recognized identical nucleotide sequences in the samples and the standard RNA. The filters were washed to a stringency of 20 mM Na_2HPO_4, pH 7.6, 1% SDS, 1 mM EDTA at 65°C and the filters subjected to autoradiography. A laser densitometer was used for quantitations (Molecular Dynamics; Image Quant version 3.1 software).

Labeling of transcripts with [^{32}P]UTP

Transcription reactions intended for pulse-labeling were prepared as described above. The 460 bp *Pvu*II fragments (10 ng) of pT8C2KS or pA8G8KS served as templates. The 211 bp *Pvu*I–*Xho*I fragment (30 ng) of pBluescript KS was used as an internal control. Pulse-labeling was carried out by mixing 10 µl transcription reaction with 40 µl labeling mixture (10 mM DTT, 0.5 mM ATP, CTP and GTP, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl, 40 mM Tris–HCl, pH 7.5, 0.12 µCi/µl [α-^{32}P]UTP, 100 ng/µl BSA, 1 U/µl RNase inhibitor, 20–100 ng *Hin*dIII-digested pBluescript KS+ fragment as a labeling control, 20 U T7 RNA polymerase) and incubating for 5 min at 37°C before recovery by precipitation (150 µl 96% EtOH, 6 µl 4 M NaCl). The labeled RNA was analyzed using 10% polyacrylamide–7 M urea sequencing gels and autoradiography.

RESULTS

Transcription-mediated PNA binding

To test whether the transcriptional elongation complex could mediate $(PNA)_2$/dsDNA strand invasion complex formation we employed T7 and T3 phage RNA polymerases *in vitro* transcription systems. Two 5′-end-labeled 460 bp *Pvu*II DNA fragments, designated A8G2KS/*Pvu*II and T8C2KS/*Pvu*II, were subjected to T3 RNA polymerase transcription in the presence of PNA 575. These templates contained single targets complementary to PNA 575 inserted in either orientation downstream of the phage promoters (Fig. 1). Following transcription the fraction of template comprising PNA strand displacement complexes was determined by $KMnO_4$ probing and gel analysis. Strand invasion was detected as a series of bands compatible with 276–286 or 174–184 nt long DNA fragments, as would result from cleavage of the displaced loops of A8G2KS/*Pvu*II or T8C2KS/*Pvu*II respectively. As shown in Figure 2, formation of the $(PNA)_2$/dsDNA strand displacement complex depended on the presence of ribonucleotides, T3 RNA polymerase and the concentration of PNA. By comparing the intensities of the transcription-dependent signals to those obtained from $(PNA)_2$/dsDNA strand displacement complexes preformed in low salt, thus representing 100% binding (Fig. 2, lanes 1 and 10), it was possible to estimate the fraction of template molecules bound by PNA. We found that transcription in the presence of 5 µM PNA 575 mediated binding of PNA to 25–75% of the template molecules within 60 min (compare lane 1 with lane 4 and lane 10 with lane 13), whereas lower concentrations of PNA resulted in lower fractions of template molecules involved in $(PNA)_2$/dsDNA triplexes. In contrast, without transcription almost none of the template molecules were bound by PNA, showing that the transcription buffer, as expected, contained sufficient Na^+, Mg^{2+} and spermidine to slow down spontanous $(PNA)_2$/dsDNA triplex formation dramatically (lanes 2, 3, 6, 7, 11, 12, 16 and 17). Thus transcription with T3 RNA polymerase could facilitate PNA binding with both the template strand and with the non-template strand (lanes 4, 8, 13 and 17). Similar results were obtained using T7 RNA polymerase (data not shown).

Kinetics and correlation with RNA synthesis

To compare the efficiencies of transcription-mediated PNA binding to templates with PNA targets situated on either of the two DNA strands equal amounts of radiolabeled A8G2KS/*Pvu*II and T8C2KS/*Pvu*II were mixed, subjected to T7 RNA polymerase transcription in the presence of 5 µM PNA 655 and after different periods of incubation probed with $KMnO_4$ (Fig. 3). The results were quantified by densitometric scanning. By comparing with signals obtained from $(PNA)_2$/dsDNA complexes preformed

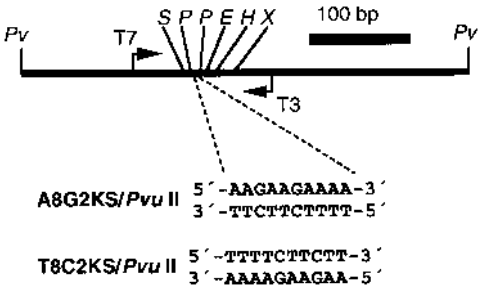

Figure 1. Schematic representation of the DNA constructs used. The T3 and T7 phage promoters are indicated by arrows. Recognition sites for *Pvu*II, *Sma*I, *Pst*I, *Eco*RI, *Hin*dIII and *Xho*I are denoted by *Pv, S, P, E, H* and *X* respectively. The orientation and sequence of the PNA targets situated in A8G2KS/*Pvu*II and T8C2KS/*Pvu*II are shown below the map.

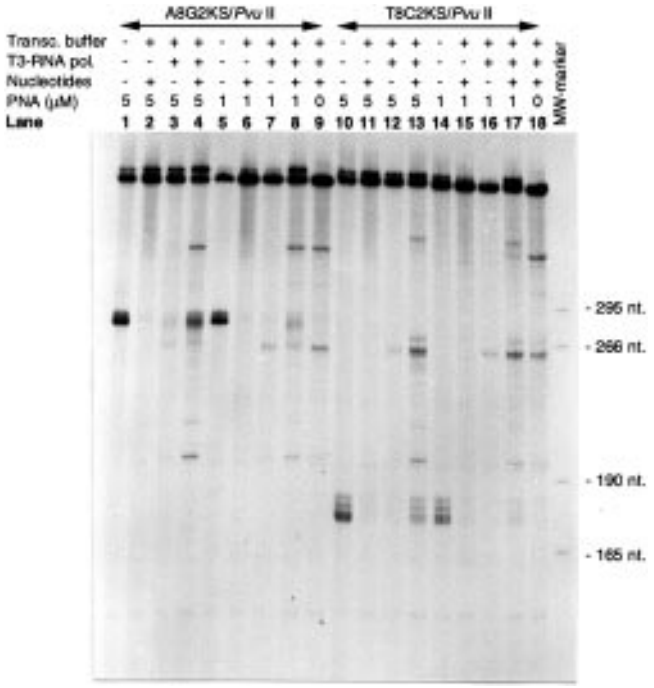

Figure 2. Transcription-mediated binding of PNA 575 to template DNA. The 5′-radiolabeled A8G2KS/*Pvu*II or T8C2KS/*Pvu*II templates were incubated for 60 min under conditions as indicated above each lane, probed with $KMnO_4$ and resolved on a 7% denaturing polyacrylamide gel. Molecular weight markers were prepared by mixing 5′-radiolabeled A8G2KS/*Pvu*II fragments digested with *Sma*I or *Eco*RI.

in low salt (lane 1) and assuming that the (identical) promoters of the two constructs directed transcription equally efficiently it was estimated that transcription-mediated PNA binding occurred approximately three times as efficiently with the non-template strand as it did with the template strand.

In order to establish a correlation between the rate of PNA binding and the rate of T7 RNA polymerase transcription from T8C2KS/*Pvu*II we examined transcription reactions containing 5 µM PNA 655 incubated for different time periods. The DNA template was probed with permanganate to monitor the appearance of strand displacement and the transcription rate was

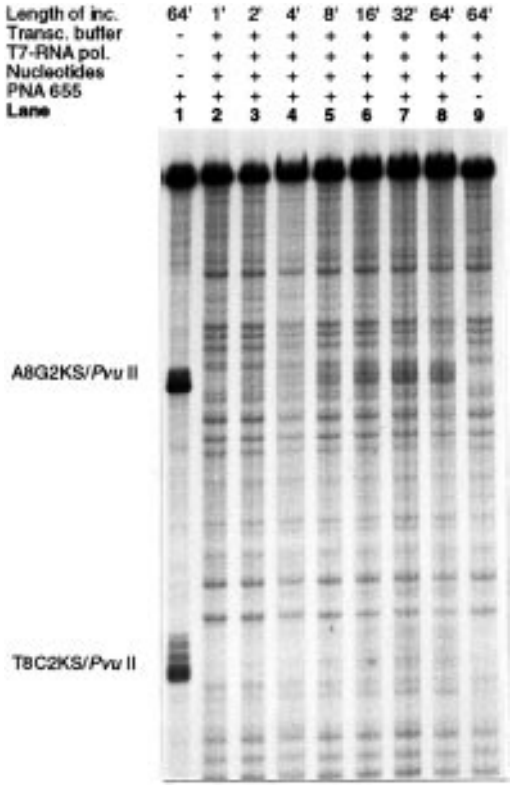

Figure 3. Binding kinetics. Equal amounts of 5′-radiolabeled A8G2KS/*Pvu*II and T8C2KS/*Pvu*II templates were mixed, subjected to T7 RNA polymerase *in vitro* transcription in the presence of 5 µM PNA 655, probed with $KMnO_4$ and finally analyzed using 7% sequencing gels and autoradiography. The lengths of incubation (min) were as indicated above each lane. The origin of the $KMnO_4$-sensitive sites are indicated to the left.

determined in parallel by slot blot analysis of the synthesized RNA using a ^{32}P-labeled fragment of pBluescript as probe (Fig. 4). From these results we calculate a PNA binding efficiency of 1% per transcription passage over the target positioned on the template strand and, consequently, we estimate that 3% of the DNA should be captured for each RNA polymerase passage when the target is situated on the non-template strand.

RNA polymerase can mediate repression of its own activity in the presence of PNA

The finding that transcription could mediate binding of PNA to the template strand under ‘high salt’ conditions implied that T8C2KS/*Pvu*II transcribed by T7 RNA polymerase in the presence of PNA could work as an *in vitro* anti-gene model system. Such transcription reactions, for which the lengths of the transcription preincubation were varied, were transferred to a labeling mixture containing [^{32}P]UTP and fresh T7 polymerase. A 211 bp *Pvu*II–*Xho*I pBluescript KS fragment lacking a PNA target was included as an internal control giving rise to a 102 nt run-off transcript. The intensities of the bands representing full-length transcripts from T8C2KS/*Pvu*II decreased as the templates were transcribed for longer time periods (Fig. 5A and B). In contrast, pulse-labeling of transcripts from the template lacking a PNA target showed only a minor reduction in the amounts of full-length transcripts (Fig. 5A and B). Similarly,

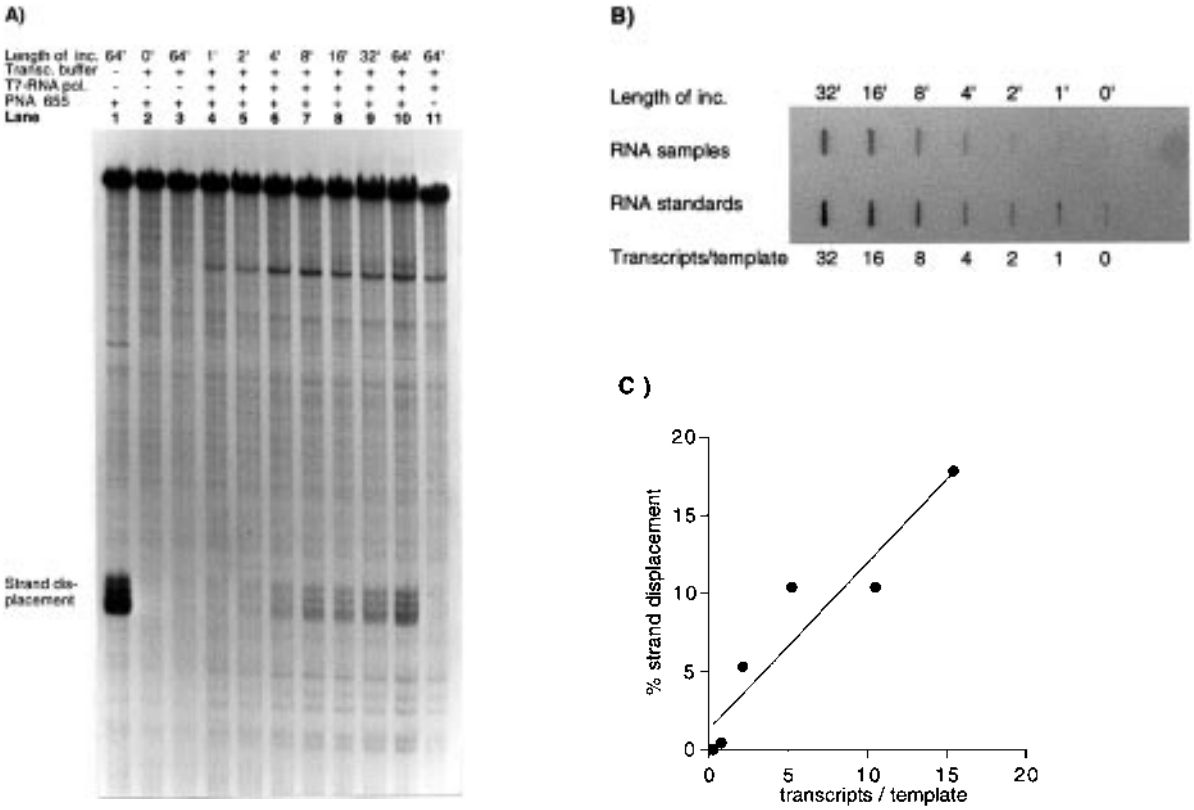

Figure 4. Correlation between the rate of $(PNA)_2$/dsDNA strand displacement and the rate of T7 RNA polymerase transcription of radiolabeled T8C2KS/*Pvu*II. (**A**) Transcription reactions containing 40 ng end-labeled T8C2KS/*Pvu*II and 5 µM PNA 655 were incubated as indicated above each lane, probed with permanganate and resolved using a sequencing gel. (**B**) Transcription rate determined by slot blot analysis of the synthesized RNA using a ^{32}P-labeled fragment of pBluescript as a hybridization probe. Lengths of transcription (min) are indicated at the top. The amounts of standard RNA loaded correspond to the indicated number of times (transcripts/templates) each DNA template should be transcribed to give a similar hybridization signal. (**C**) Graphic representation of the results obtained in (A) and (B) quantitated by laser scanning densitometry.

corresponding reactions lacking either PNA 655 or T7 RNA polymerase gave only a small reduction in the amount of T8C2KS/*Pvu*II run-off transcripts (Fig. 5B and data not shown). These results indicated that T7 transcription of T8C2KS/*Pvu*II in the presence of PNA mediated the formation of template strand-associated $(PNA)_2$/DNA triplexes which subsequently blocked further transcription. Thus the action of the polymerase mediated repression of its own activity, a process that may be regarded as 'suicide transcription'.

Notably, PNA bound to the template strand did not result in a band compatible with transcriptional arrest at the PNA target (Fig. 5A and D). This could suggest that DNA fragments involved in $(PNA)_2$/dsDNA triplexes were 'deactivated' or in other ways inaccessible to RNA polymerase. To test whether such DNA molecules bound by PNA could still serve as templates for transcription we examined T7 polymerase transcription reactions in which A8G2KS/*Pvu*II served as a template. Since the PNA target in this system is situated on the non-template strand, binding of PNA was not expected to result in transcriptional arrest of T7 RNA polymerase (13). Pulse-labeling using templates from these reactions with T3 RNA polymerase and [^{32}P]UTP resulted in bands compatible with transcriptional arrest at the PNA target (Fig. 5C and E). Quantification of the data by densitometric scanning analysis substantiated that the majority of the A8G2KS/*Pvu*II fragment remained capable of being transcribed by T3 RNA polymerase (not shown). Thus the lack of bands indicative of transcriptional arrest at the PNA target observed in Figure 5A may be due to blocking of the DNA template by accumulation of previously arrested RNA polymerases between the T7 promoter and the PNA target before transcription proceeded in the presence of [^{32}P]UTP (Fig. 5D).

DISCUSSION

The ability of PNA to interact sequence specifically with dsDNA has found a variety of applications. PNA has been demonstrated to be capable of acting as a sequence-specific inhibitor of restriction enzyme cleavage (19), as an electron microscopy marker of homopurine tracts (20), as a gene activating agent, i.e. an artificial transcription factor (21), as an inhibitor of transcription elongation (12–14) and, in cooperation with single-strand-specific nucleases, as a 'synthetic restriction enzyme' (22). However, to bypass the inhibitory effects of salts on strand displacement all such previous experiments have involved the use of $(PNA)_2$/dsDNA complexes preformed in low salt.

In the present study we show that salt inhibition of PNA strand invasion caused by the transcription buffer can be circumvented by transcriptional activity of T7 and T3 phage RNA polymerases

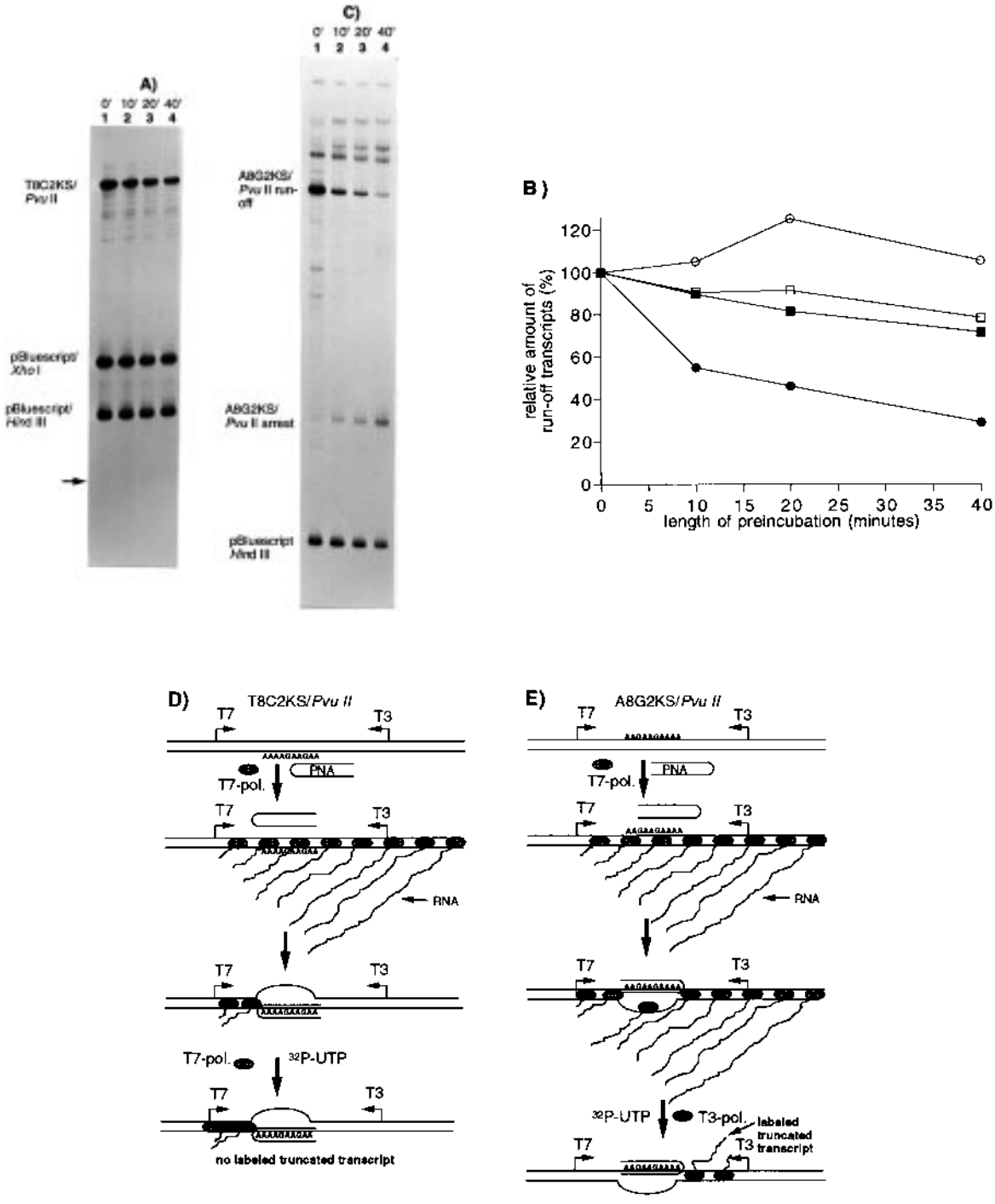

Figure 5. Suicide transcription. (**A**) *In vitro* transcription using T7 RNA polymerase and T8C2KS/*Pvu*II as a template was carried out in the presence of 10 µM PNA 655. As an internal standard a 211 bp *Pvu*II–*Xho*I fragment of pBluescript KS was added. After incubating for the periods indicated above each lane the templates were transferred to [α-^{32}P]UTP labeling reactions in which a *Hin*dIII pBluescript KS fragment was included as a labeling control. T7 RNA polymerase transcription was allowed to continue for an additional 5 min before analyzing the transcripts by denaturing polyacrylamide gel electrophoresis and autoradiography. The origins of the [^{32}P]UTP-labeled species are indicated to the left. The position where bands might be expected as a result of transcriptional arrest is indicated (arrow). (**B**) Graphic representation of the data shown in (A) and of data not shown (analogous experiments as described above except that the addition of T7 RNA polymerase or PNA 655 was omitted) quantitated by laser scanning densitometry. The presence or absence of T7 RNA polymerase and PNA 655 are indicated in brackets: (●), T8C2KS/*Pvu*II, [+Pol, +PNA]; (■), *Pvu* II/*Xho*I of pBluescript [+Pol, +PNA]; (○), T8C2KS/*Pvu*II, [–Pol, +PNA];(□) , T8C2KS/*Pvu*II, [+Pol, –PNA]. (**C**) T3 RNA polymerase *in vitro* transcription of A8G2KS/*Pvu*II involved in $(PNA)_2$/dsDNA triplexes mediated by T7 RNA polymerase transcription. *In vitro* transcription using T7 RNA polymerase and A8G2KS/*Pvu*II as a template was carried out in the presence of 10 µM PNA 655. After incubating for the periods indicated above each lane the templates were transferred to [α-^{32}P]UTP labeling reactions containing T3 RNA polymerase and *Hin*dIII-cut pBluescript KS as a labeling control. After 5 min labeling the transcripts were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. The origins of the radioactive bands are indicated to the left. (**D**) Schematic representation explaining the experiment shown in (A) and its interpretation. (**E**) Schematic representation explaining the experiment shown

as this accelerated the formation of $(PNA)_2$/dsDNA complexes dramatically. From an anti-gene point of view it is noteworthy that such transcription-mediated PNA invasion was possible when the PNA target was placed on the template strand (although not as efficient as when the target was placed on the non-template strand). As the transient transcription bubble is the only obvious single-stranded region of a transcribed linear DNA template and keeping in mind that strand invasion requires DNA 'breathing', we find it most likely that transcription-mediated triplex formation is catalyzed by the transcription bubble itself. This is supported by our finding that strand invasion occurred more efficiently when the PNA target was placed on the non-template strand as PNA binding to the template strand of the transcription bubble would be expected to involve competition with the nascent RNA chain (15). We cannot formally exclude the possibility that binding of PNA is caused by other phenomena than the transcription bubble. We anticipate that the activity of multi-subunit polymerases, e.g. eukaryotic RNA polymerase II, will mediate PNA strand displacement similarly to that observed for the phage polymerases and thus the findings reported here may be highly relevant for the possible future use of PNA as a gene-targeted drug *in vivo*. Since only genes which are transcribed could be considered relevant targets for anti-gene therapy, the use of transcription to mediate sequence-specific PNA binding does not limit the number of potential target genes.

Alternative approaches for modulating the activity of endogenous gene expression, such as the use of antisense oligonucleotides or transgene expression of antisense RNA or ribozymes, suffers from the possibility that the targeted gene may be up-regulated as a consequence of a reduced level of gene product. In contrast, if using suicide transcription mediated by PNA then up-regulation of gene expression should result in even more efficient repression. Therefore, suicide transcription is, in theory, an extremely attractive approach when the task is to shut down expression from feedback-regulated genes.

The results shown here also imply that PNA offers a unique opportunity for using strand displacement to monitor transcription, thus making possible detection of the activity of RNA polymerase by examining the templates instead of the transcripts. This could be suitable for studying *in vitro* transcription using extracts in which RNase contamination is a problem or, by using biotinylated PNA, for determining which of multiple templates, each giving rise to identical transcripts, are expressed. Finally, this method could, in principle, be used to determine the relative lifetimes of transcription bubbles at specific sites along a transcribed DNA sequence. However, the sequence restrictions of triplex formation would have to be overcome before this aspect of the present results can be fully explored.

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