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ACKNOWLEDGEMENT. The work was supported by a grant from the Department of Biotechnology, Government of India

Received 14 August 1990, revised accepted 30 October 1990

Alteration in the '-10' sequence of the A2 promoter of bacteriophage T7 reduces the rate of transcription initiation

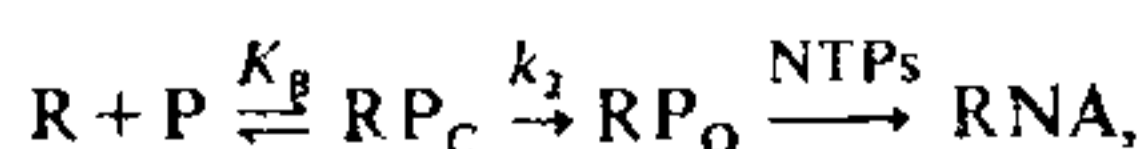
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The rate of transcription initiation, in the absence of other factors, is determined by intrinsic properties of the promoter. This largely depends on the DNA structure at the promoter region. The A2 promoter of bacteriophage T7 is an early promoter, specific for the RNA polymerase of the host *E. coli*. It is also one of the strongest promoters tested so far *in vitro*. We show here that a mutation in the '-10' region of T7A2 only reduces the strength of the promoter: transcription driven by the mutant promoter results in much less product. The site of initiation is the same as that in the wild-type promoter.

ANALYSIS of the various promoter sequences recognized by the major form of *E. coli* RNA polymerase, $E \delta^{70}$, has shown that there are two highly conserved hexanucleotide sequences, TTGACA and TATAAT, centred around 35 and 10 base pairs upstream from the start point of RNA synthesis, and designated '-35' and '-10' boxes respectively¹⁻³. One important feature of the promoter sequence is that it is asymmetric, and this determines the initiation of transcription in a fixed direction on the correct (template) DNA strand⁴.

RNA chain-initiation frequencies in *E. coli* vary over a range of about one thousand-fold⁵. One of the important questions is how RNA polymerase recognizes the DNA sequences at the promoters around the -35 and -10 regions in a manner that results in a three-orders-of-magnitude variation in reaction rate⁶. The model proposed by Chamberlin⁷ for the interaction of promoter with RNA polymerase is well accepted now, and can be depicted as follows:



where R is the enzyme, P represents promoter DNA, and RP_C and RP_O designate two intermediate states, viz. the closed and open complex respectively. McClure and coworkers^{6,8} correlated promoter strength with these two constants K_B and k_2 , and the relationship is generally true of all the promoters studied so far.

Promoter mutation studies have indicated that the mutations affect the rate of initiation of transcription by altering either K_B or k_2 . These mutations fall in the -35 or the -10 region^{9,10}. These studies generally suggested that the -35 region is mainly involved in polymerase binding whereas the -10 region plays a major role in the isomerization of the closed complex to the open complex.

Bacteriophage T7 has three major early promoters A1, A2 and A3, which are recognized by the RNA polymerase of the host *E. coli*. These promoters are known to be strongest *in vitro*⁶. We have used these promoters, individually cloned in the *Bam*HI site of pBR322 (obtained from F. W. Studier, Brookhaven National Laboratory, USA), in studies on promoter-RNA polymerase interaction¹¹⁻¹³. In the presence of T7 early promoters, *E. coli* RNA polymerase transcribes almost exclusively from them, without recognizing the much weaker pBR322 promoters, even at high enzyme/DNA ratio (ref. 14, and A. Rosenberg, personal communication). When these promoters are cloned in pBR322 (Figure 1), and the plasmid digested with *Sal*I, as in the present case, the lengths of the run-off transcripts are expected to be 340 base pairs at T7A1, 310 at T7A2, and 305 at T7A3.

Of particular interest in this communication is the promoter T7A2. The sequence of the T7A2 promoter is given below:

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CGAAAAACAGGTATTGACAACA
                    -35
TGAAGTAACATGCAGTAAGATA
                    -10
CAAATCGCTAGGTAACA
                    +1
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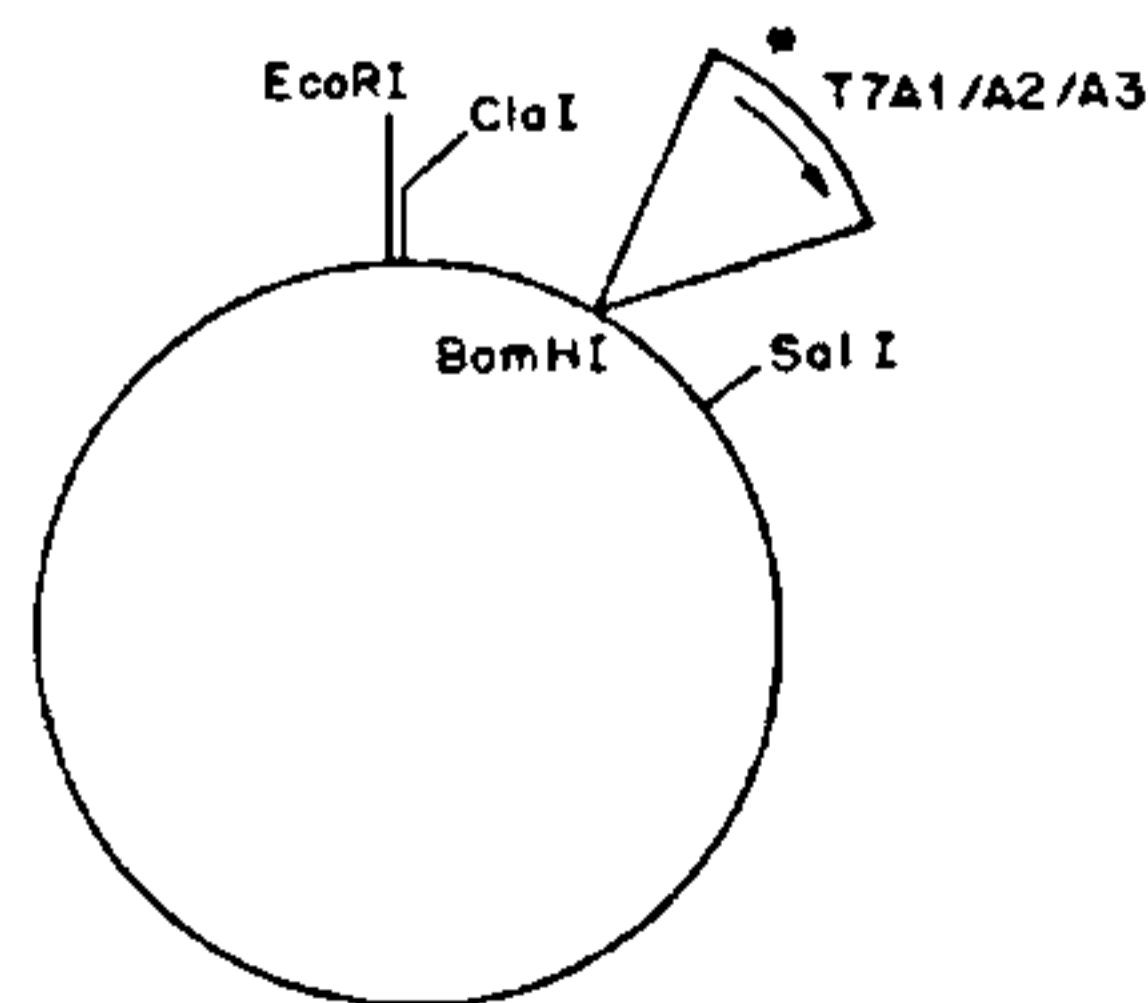


Figure 1. Diagram showing the position and orientation of the T7 promoter inserts in pBR322. The arrow indicates the direction of transcription from the promoter, which is marked by an asterisk.

The pBR322-derived plasmid in which the T7A2 promoter has been cloned is named pAR1539. From our stock of pAR1539, we obtained a clone which yielded much less product in a run-off transcription reaction, although the product was of the same size as that obtained from the original pAR1539. Figure 2 shows such run-off transcripts obtained from original as well as presumably altered T7A2, along with transcripts obtained from T7A1 and T7A3 promoter-containing plasmids used as controls. It appeared that pAR1539 had undergone a spontaneous mutation on storage, resulting in less efficient transcription at T7A2. The difference in transcription efficiency necessitated the sequencing of the promoter DNA in the clone. Figure 3 shows that the A2 promoter insert in this clone has a three-base substitution in the -10 box, i.e. AGC in

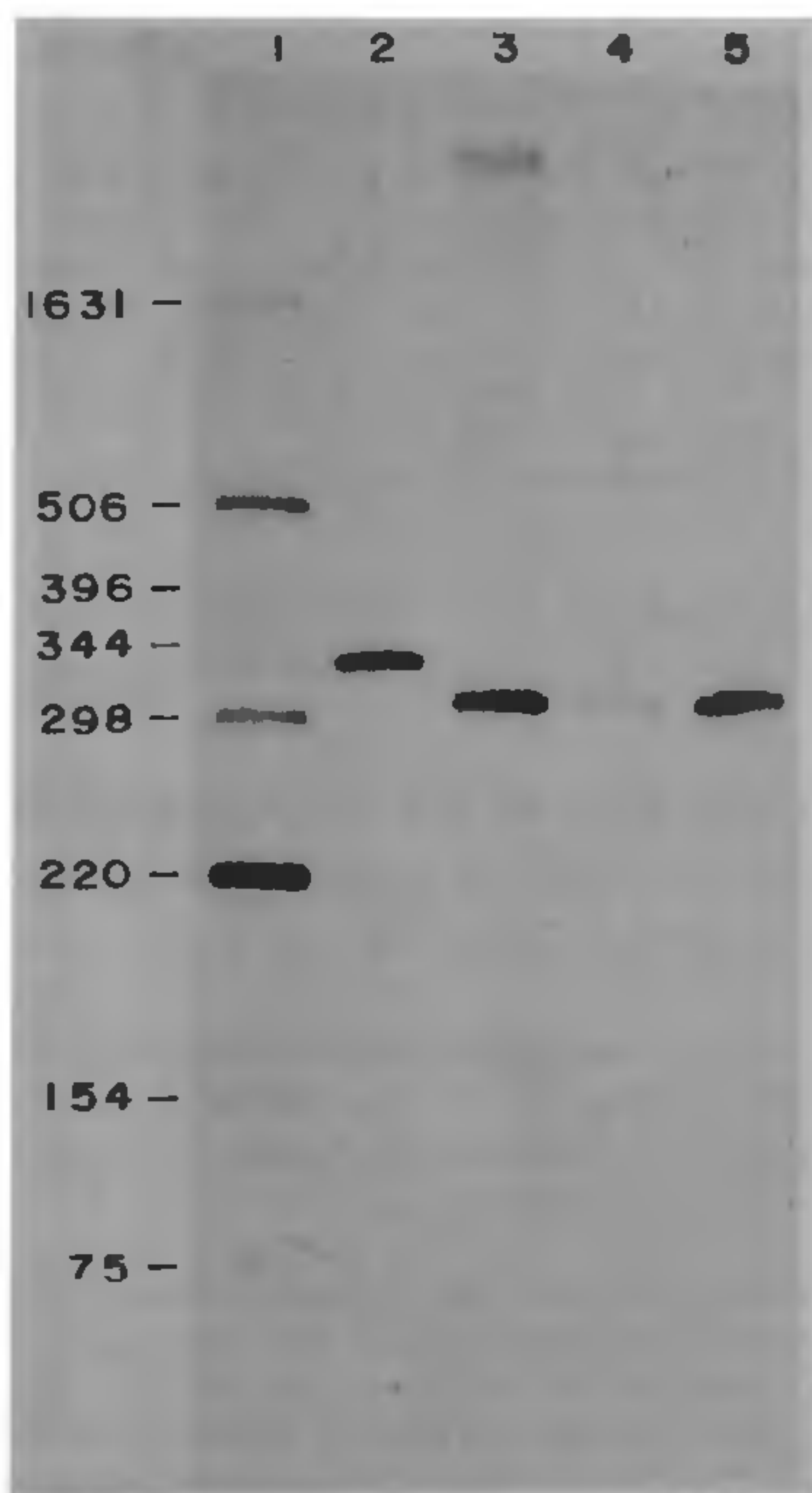


Figure 2. Run-off transcription from bacteriophage T7 early promoter-containing plasmids. One μg of plasmid DNA was linearized with *Sall* and used in a transcription reaction containing 0.4 mM NTPs and 0.1 mM $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (1 $\mu\text{Ci}/\text{nmol}$), 40 mM Tris-Cl (pH 7.9), 10 mM MgCl_2 , 150 mM KCl, 0.2 mM DTT, 0.1 mM EDTA, 1 μg (3 units) of *E. coli* RNA polymerase and 2 units of RNasin. The mixture was incubated at 37°C for 15 min in a total reaction volume of 20 μl . Five microlitres of the reaction mixture was then incubated with 3 μl of formamide loading buffer in a boiling water bath for 3 min, chilled, and electrophoresed in a 4% polyacrylamide gel containing 8 M urea at 350 V with 1X TBE. Lane 1, molecular weight markers, and run-off transcripts from: lane 2, T7A1, lane 3, T7A2; lane 4, T7A2*; lane 5, T7A3.

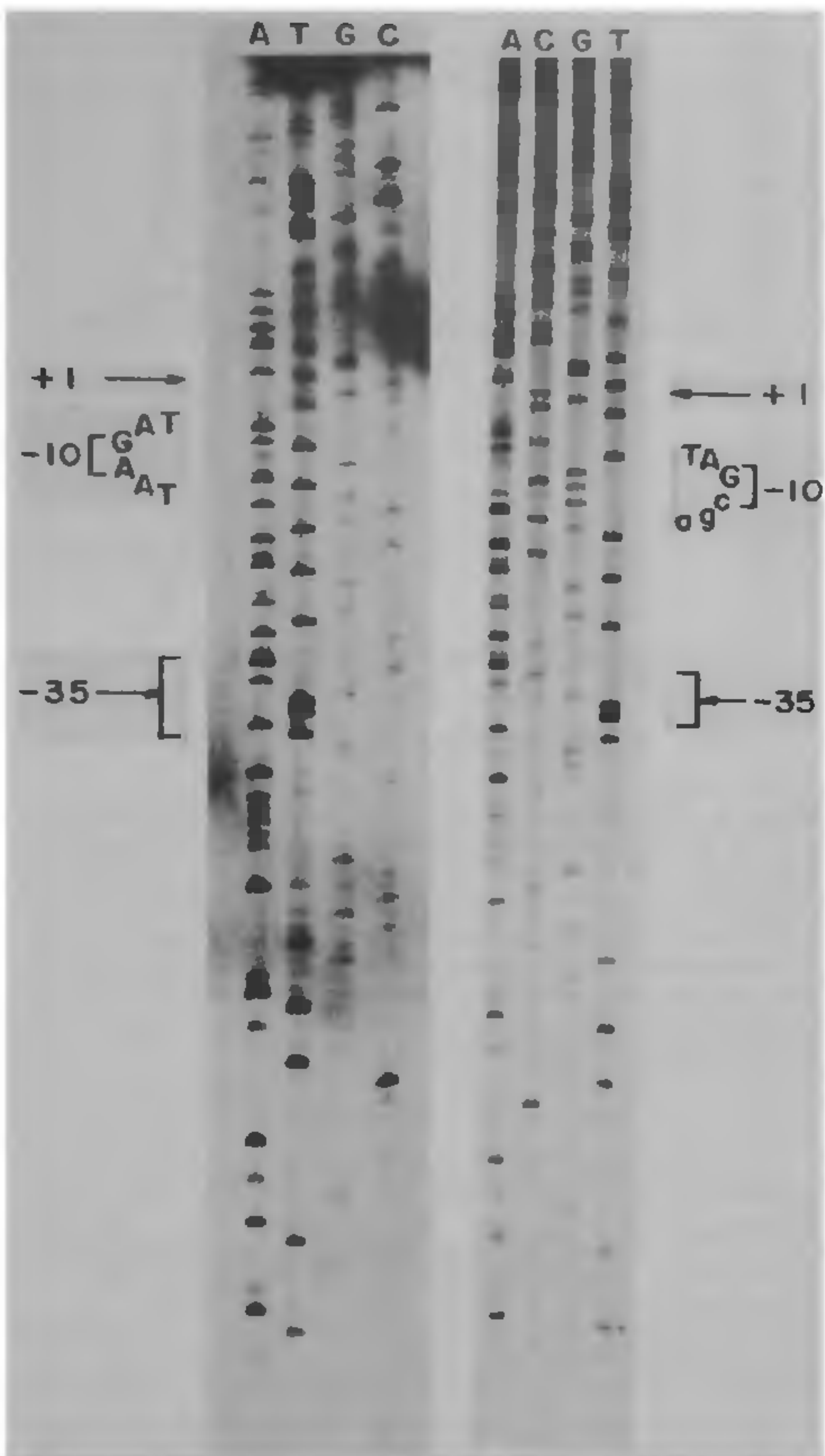


Figure 3. Dideoxy sequencing of T7A2 (left) and T7A2* (right). The positions of the mutations in T7A2* are indicated by lower-case nucleotide symbols.

place of the TAA of the wild-type A2 promoter. The mutant promoter was designated T7A2*, and the plasmid containing the mutant promoter pAR1539*. This three-base substitution has considerably reduced the frequency of transcription initiation at the A2* promoter. However, the site of transcription initiation remains unaltered, as evidenced from abortive-initiation reactions carried out with T7A2 and T7A2*.

Under the conditions of abortive initiation of transcription¹⁵, when only the first two nucleotides corresponding to the 5' initiation site of the RNA message were supplied, both T7A2 and T7A2* turned over pppGpC, a dinucleotide product, as shown in Figure 4. One can notice from the figure that the mobility of the slower-moving peak (the dinucleotide

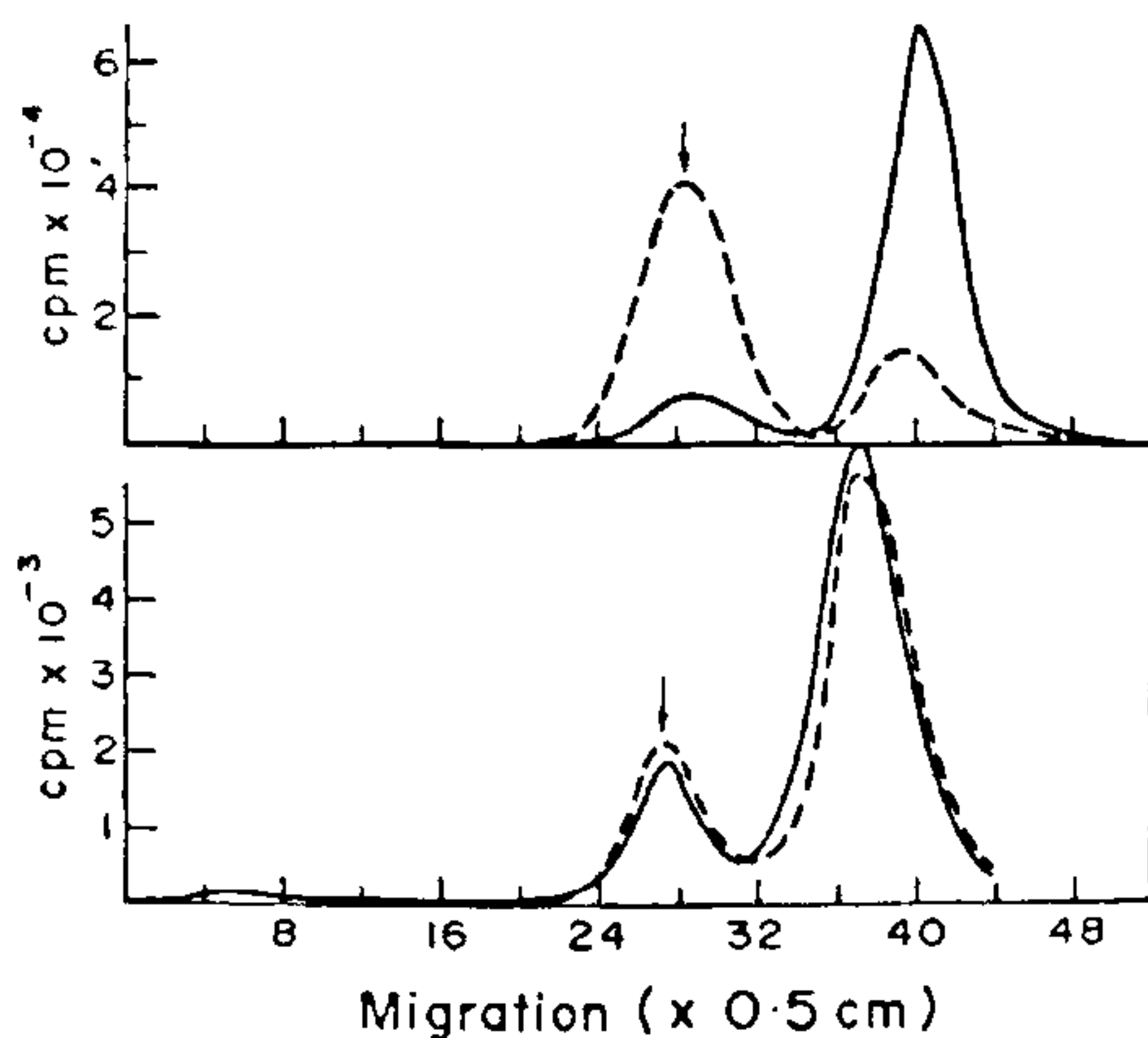


Figure 4. Paper-chromatographic analysis of abortive-initiation reaction for the synthesis of dinucleotide tetraphosphate pppGpC from T7A2 (top) and T7A2* (bottom) promoters in the absence of rifampicin (—) and in the presence of $50 \mu\text{g ml}^{-1}$ rifampicin (---). In both cases, fraction 1 represents origin, and the first peak from the left corresponds to the product and the second peak represents unincorporated nucleotide. The concentration of GTP was $400 \mu\text{M}$, [$\alpha\text{-}^{32}\text{P}$]CTP was $3 \mu\text{M}$ ($1 \mu\text{Ci/nmol}$), DNA was 0.4 mM DNA phosphorus, with $0.16 \mu\text{M}$ RNA polymerase.

product) is the same in the two cases. This was further confirmed by treating the product with alkaline phosphatase and verifying comigration of the dephosphorylated product with commercial GpC on a polyethylenimine plate. Thus it is evident that the mutation in the -10 box did not alter the recognition of the initiation site by RNA polymerase. We have noticed recently¹⁶ that rifampicin, which is a classical inhibitor of initiation of transcription in *E. coli*¹⁷, stimulates the abortive synthesis of dinucleotide tetraphosphate at the T7A2 promoter (Figure 4). It has been observed that the product pppGpC forms a stable ternary complex at A2, resulting in poor turning over of the dinucleotide. However, in the presence of rifampicin, the complex is destabilized, and as a consequence rapid abortive synthesis continues and the product accumulates¹⁶. The mechanism of this effect will be discussed elsewhere. The interesting point to note here is that, even with T7A2*, rifampicin causes enhancement of the abortive synthesis of GpC, although to a much smaller extent. We have observed this stimulation repeatedly and therefore consider it real and not an experimental artifact. Interestingly, at T7A2* the stability of the ternary complex is lower than that at T7A2. It is this that has resulted (Figure 4, bottom) in the turning over and accumulation of pppGpC to a significantly greater extent with T7A2* than with T7A2.

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Received 22 August 1990; revised accepted 20 November 1990

Microinjection of rat growth-hormone gene into zebrafish egg and production of transgenic zebrafish

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A plasmid containing rat growth-hormone gene was microinjected into fertilized zebrafish eggs prior to first cleavage; survival of the embryos one day after the injection averaged 46%. Genomic DNA extracted from presumptive transgenic fish was analysed by slot-blot and Southern-blot hybridization using labelled plasmid as probe. Patterns of hybridization indicated genomic, extrachromosomal, as well as mosaic integration. The concentration of persisting extrachromosomal DNA progressively decreased in F_1 and F_2 generations. The growth rate of transgenic fish was higher in F_0 and F_1 generations, but lower in F_2 , which may be due to transient expression of extrachromosomal DNA carrying the growth-hormone gene in F_0 and F_1 generations.

GENE-transfer technology has become a powerful tool