Kondo, H., & Ishiwata, S. (1976) *J. Biochem. (Tokyo) 79*, 159–171.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maruta, H., & Isenberg, G. (1983) *J. Biol. Chem. 258*, 10151–10158.
Maruyama, K., Kimura, S., Ishii, T., Kuroda, M., Ohashi, K., & Muramatsu, S. (1977) *J. Biochem. (Tokyo) 81*, 215–232.
Neuhaus, J.-M., Wanger, M., Keiser, T., & Wegner, A. (1983) *J. Muscle Res. Cell Motil. 4*, 507–527.
Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10–21.
Pollard, T. D., & Mooseker, M. S. (1981) *J. Cell. Biol. 88*, 654–659.
Rees, M. K., & Young, M. (1967) *J. Biol. Chem. 242*, 4449–4458.
Southwick, F. S., & Hartwig, J. H. (1982) *Nature (London) 297*, 303–307.
Tellam, R., & Frieden, C. (1982) *Biochemistry 21*, 3207–3214.
Tilney, L. G., Bonder, E. M., Coluccio, L. M., & Mooseker, M. S. (1983) *J. Cell. Biol. 97*, 112–124.
Tobacman, L. S., & Korn, E. D. (1982) *J. Biol. Chem. 257*, 4166–4170.
Walsh, T. P., Weber, A., Higgins, J., Bonder, E. M., & Mooseker, M. S. (1984) *Biochemistry 23*, 2613–2621.
Wang, Y., & Taylor, D. L. (1981) *Cell (Cambridge, Mass.) 27*, 429–436.
Wegner, A. (1976) *J. Mol. Biol. 108*, 139–150.
Wegner, A. (1982) *J. Mol. Biol. 161*, 607–615.
Wegner, A., & Neuhaus, J.-M. (1981) *J. Mol. Biol. 153*, 681–693.
Wegner, A., & Isenberg, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4922–4925.
Woodrum, D. T., Rich, S. A., & Pollard, T. D. (1975) *J. Cell Biol. 67*, 231–237.
Yamamoto, K., Pardee, J. E., Reidler, J., Stryer, L., & Spudich, J. A. (1982) *J. Cell Biol. 95*, 711–719.
Yin, H. L., & Stossel, T. P. (1979) *Nature (London) 281*, 583–586.

Efficiency of in Vitro Transcription of *Dictyostelium discoideum* Actin Gene Is Affected by the Nucleotide Sequence of the Transcription Initiation Region†

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ABSTRACT: The actin gene of *Dictyostelium discoideum* is transcribed faithfully but with very low efficiency in a cell-free system containing HeLa cell extract [Takiya, S., Tabata, T., Iwabuchi, M., Hirose, S., & Suzuki, Y. (1984) *J. Biochem. (Tokyo) 95*, 1367–1377]. Using the same in vitro system, we determined that the promoter activity of the actin 5 gene is 100–200 times weaker than that of the silkworm fibroin gene. To clarify the cause of the low transcription efficiency, various chimeric genes were constructed from the actin and fibroin genes, and their transcription efficiencies were examined in vitro. Both the TATA box and the transcription initiation site of the two natural genes functioned in the transcription of the chimeric genes, the efficiency of which was especially affected by the transcription initiation region. In chimeric genes having the upstream sequence of the actin gene and a downstream sequence including the transcription initiation site of the fibroin gene, the transcription efficiency was higher than one-third of that of the natural fibroin gene. In chimeric genes having the actin transcription initiation region and an upstream sequence of the fibroin gene, the transcription efficiency was as low as that of the natural actin gene. We concluded that the transcription initiation site is a part of the promoter and an essential region for directing faithful and efficient initiation of gene transcription.

Attempts to reveal the promoter region of eukaryotic class II genes have yielded information about nucleotide sequences essential for transcription. One conserved sequence, the TATA box, in the 5′-flanking region of the class II genes is indispensable for accurate and efficient gene transcription (Corden et al., 1980; Grosschedl et al., 1980; Wasylyk et al., 1980; Grosveld et al., 1981; Tsujimoto et al., 1981; Hirose et al., 1982; Tokunaga et al., 1984). Other functional elements promoting or modulating transcription have been found in the region surrounding the TATA box or far upstream from it (Melon et al., 1981; Tsuda & Suzuki, 1981, 1983; Grosschedl & Birnstiel, 1982). However, inconsistent results have been reported on the role of the transcription initiation site (Corden et al., 1980; Tsujimoto et al., 1981; Grosveld et al., 1981; Dierks et al., 1983). Of these functional elements, the TATA

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box is considered to be essential for transcription of the class II genes, especially in vitro. Deletion or substitution of any nucleotides in the TATA box may abolish transcription or decrease its efficiency, while deletion of the far upstream sequences does not affect gene transcription in a cell-free system containing HeLa cell extract, except for several viral genes (Myers et al., 1981; Hen et al., 1982; Lee et al., 1982) and sea urchin histone H2A gene (Grosschedl & Birnstiel, 1982). The TATA box is thought to be necessary for efficient initiation of transcription at a particular site about 30 base pairs (bp)[1] downstream from it both in vitro and in vivo.

McKeown & Firtel (1981), who reported that *Dictyostelium discoideum* actin genes form a multigene family consisting of about 17 members (Kindle & Firtel, 1978; Firtel et al., 1981), showed that almost all the actin genes have a TATA boxlike sequence, comparable to the canonical sequence of the TATA box seen in other class II genes, around 30 bp upstream from the transcription initiation site. However, the regions surrounding this sequence of the actin genes differ from those of many other class II genes. For example, there is a characteristic T stretch just downstream of the TATA boxlike sequence, and the 5′-flanking sequences of these genes are unusually AT rich (90–95%) (Firtel et al., 1979; Kindle & Firtel, 1981). Thus, it is of interest to know how such characteristic sequences near the promoter region influence the transcription efficiency of actin genes.

We previously showed that the actin 5 and 6 genes were faithfully transcribed from the in vivo transcription initiation site in a cell-free system containing HeLa cell extract (Takiya et al., 1984). However, the transcription efficiency was extremely low, compared with that of the Ad2 major late gene in the same system. In the present study, to find the cause of the low transcription efficiency of the actin genes in vitro and to define the signal sequence promoting transcription of these genes, we constructed a variety of chimeric genes from the actin 5 gene and the silkworm fibroin gene that is efficiently transcribed in the HeLa cell extract and then investigated the in vitro efficiency of faithful initiation of transcription of these chimeric genes. The experimental results suggested that the TATA box and its upstream sequence are exchangeable between the actin and fibroin genes; the chimeric genes that had been constructed by exchanging the upstream sequence including the TATA box exhibited transcription as strong as that of the natural genes. On the other hand, the promoter activity of the chimeric gene that had the downstream sequence including the transcription initiation region of the actin or fibroin gene was greatly affected by the intrinsic activity of the promoter of the natural gene from which the transcription initiation sequence had been obtained.

Materials and Methods

Construction of Deletion Mutant and Chimeric Genes. The chimeric genes were constructed by reciprocal exchange of the upstream and downstream sequences of the presumed promoter regions of the actin and fibroin genes. For this purpose, several 5′- and 3′-deletion mutants of the actin 5 gene (pDd actin 5; provided by Dr. R. A. Firtel) were first prepared. The corresponding 5′- and 3′-deletion mutants of the silkworm fibroin gene were obtained from Tsujimoto et al. (1981). Figure 1 shows the strategy for constructing the chimeric genes. The pDd actin 5 was digested with *Mbo*II to obtain a 1.6-kb fragment containing the actin promoter region (−1500 to

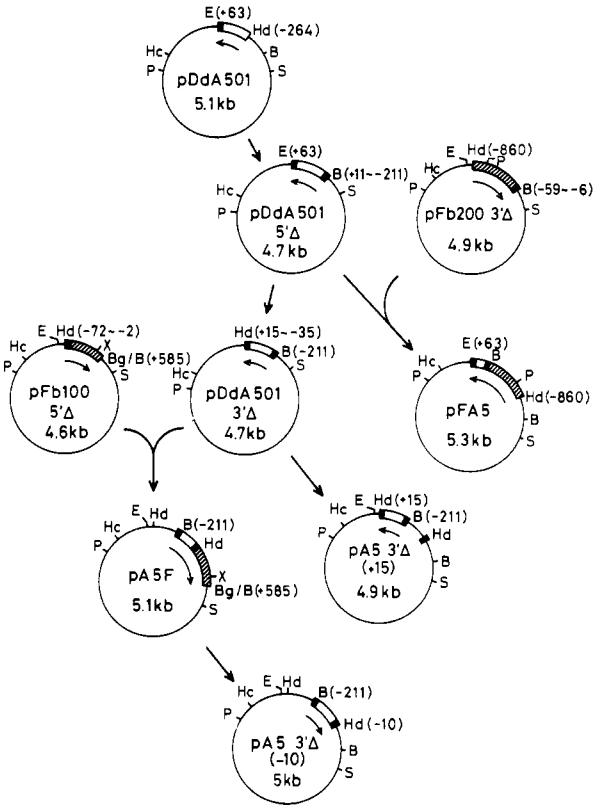

FIGURE 1: Recombinant plasmids of the deletion mutants of *D. discoideum* actin 5 and *B. mori* fibroin genes and the strategy for constructing chimeric genes. Details of the construction of the recombinant plasmids are described under "Materials and Methods". Open and hatched boxes indicate the sequences of the actin and fibroin genes containing the 5′-flanking region, respectively. The black boxes indicate synthetic nucleotide linkers, the thin lines, pBR322 or pBR325, and the arrows, the direction of transcription of the actin or fibroin gene. Restriction sites relevant to the reconstruction of the recombinant plasmids are shown, and the position number of the restriction sites relative to the transcription initiation point (+1) of the actin and fibroin genes is indicated in parentheses. B, *Bam*HI; E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

~+65). Both ends of the *Mbo*II fragment were flushed by S1 nuclease digestion and joined to an *Eco*RI linker, followed by treatments with *Eco*RI and *Hin*dIII to produce a 330-bp fragment containing the sequence from −264 to +63 (two nucleotides were removed by S1 digestion). The 330-bp fragment was inserted into pBR325 in place of the *Eco*RI/*Hin*dIII fragment of pBR325 (pDdA501 in Figure 1). The pDdA501 was linearized with *Hin*dIII and digested with nuclease *Bal*31 to produce a series of 5′-deletion mutants. The nibbled ends were ligated with a *Bam*HI linker, and the deleted pDdA501 was circularized (pDdA501 5′Δ in Figure 1). To obtain 3′-deletion mutants of the actin gene (pDd actin 3′Δ), the recombinant plasmid carrying the 5'-deletion mutant, pDdA501 5′Δ(−211) [the minus number in parentheses represents the nucleotide position upstream from the in vivo transcription start point (+1) on the noncoding strand of the actin gene], was cleaved with *Eco*RI, treated with nuclease *Bal*31 for various periods, and circularized after ligation of a *Hin*dIII linker to both deleted ends (pDdA501 3′Δ in Figure 1).

To construct chimeric genes of the pFA5 series, the *Bam*HI/*Pst*I fragment was prepared from the pDdA501 5′Δ mutant and linked to the *Hin*dIII/*Bam*HI fragment from the pFb200 3′Δ at the *Bam*HI site. The joined fragment was double digested with *Hin*dIII and *Eco*RI to produce a fragment about 920 bp long, which had the fibroin gene sequence

[1] Abbreviations: bp, base pair(s); kb, kilobase(s); kbp, kilobase pair; Ad2, adenovirus 2; SV40, simian virus 40.

(to −860) in the upstream side of the joining site and the actin gene sequence (to +63) in the downstream side, and then introduced to the *Eco*RI/*Hin*dIII region of pBR322 for recloning. A series of the reverse type of chimeric genes, the pA5F series, was constructed from the 3′-deletion mutant (pDdA501 3′Δ) of the actin 5 gene and the 5′-deletion mutant (pFb100 5′Δ) of the fibroin gene. In this case, the *Sal*I/*Hin*dIII fragment was isolated from the pDdA501 3′Δ and joined to the *Hin*dIII/*Sal*I fragment from the pFb100 5′Δ at the *Hin*dIII site. The resultant *Sal*I/*Sal*I fragment was digested with *Bam*HI to produce the 1.1-kb *Sal*I/*Bam*HI fragment partly containing the actin and fibroin sequences. This fragment was inserted into pBR322 instead of the *Sal*I/*Bam*HI fragment of this vector for recloning.

The recombinant plasmid, pA5 3′Δ(+15), which has the −211 to +15 sequence of the actin 5 gene, was made from pDdA501 3′Δ(+15) with the introduction of an additional *Hin*dIII site to its *Sal*I sequence by ligating the *Hin*dIII linker after enzymatic collapse of the *Sal*I sequence. The modified pDdA501 3′Δ was digested with *Hin*dIII to obtain the fragment (ca. 500 bp) that contained the actin promoter region, and the resulting fragment was then recloned at the *Hin*dIII site of pBR322. The recombinant plasmid, pA5 3′Δ(−10), carrying the −211 to −10 sequence of the actin 5 gene, was derived from the pA5F(−10)(−2). The *Hin*dIII fragment containing the −211 to −10 sequence of actin 5 gene was prepared from the pA5F(−10)(−2) treated with *Hin*dIII and recloned at the *Hin*dIII site of pBR322.

The recombinant plasmids obtained at each step of gene manipulation were transfected into *Escherichia coli* HB101 and recloned, and the plasmid DNA was prepared as described previously (Takiya et al., 1984).

Both the orientation and the structure of the deletion mutant and the chimeric genes introduced into the plasmid were checked by the restriction endonuclease and sequencing analyses according to Maxam & Gilbert (1980).

In Vitro Transcription. In vitro transcription was carried out as described previously (Takiya et al., 1984), and transcripts were analyzed according to the procedure of Tsujimoto et al. (1981). HeLa cell extract was prepared by the method of Manley et al. (1980) with slight modifications.

To determine the in vitro efficiency of faithful initiation of transcription of cloned genes using truncate templates, the autoradiogram of an [α-^{32}P]UTP-labeled transcript was densitometrically scanned, and the data were corrected in the light of the UMP content which is inferred from the sequence data of the template. Then we calculated the molar concentrations of templates required to produce the same moles of transcripts as given by the truncate template from pFb200 3′Δ(+10) having the normal fibroin gene promoter. The relative efficiency of in vitro gene transcription was expressed as the reciprocal of the molar concentration of templates which was calculated above (see Figure 9).

Enzymes and Radioisotopes. Restriction endonucleases, bacterial alkaline phosphatase, and T4 polynucleotide kinase were purchased from Takara Shuzo, Kyoto, Japan; T4 DNA ligase and nuclease *Bal*31 were from Bethesda Research Laboratories Inc; synthetic *Eco*RI and *Bam*HI linkers were from New England Biolabs, Inc; *Hin*dIII linker was from Genex Co. [α-^{32}P]UTP and [γ-^{32}P]ATP were obtained from Amersham.

RESULTS

Comparison of the in Vitro Efficiency of Faithful Transcription between Actin and Fibroin Genes. We previously reported that the efficiency of faithful initiation of transcription of the *D. discoideum* actin 5 gene was very low in the in vitro system using HeLa cell extract compared with the Ad2 major late gene (Takiya et al., 1984). Tsujimoto et al. (1981) indicated that the cloned fibroin gene of *Bombyx mori* was as active as the Ad2 major late gene when used as a template in the HeLa cell extract. To find the cause of the difference between the actin and fibroin genes in the transcription efficiency, we compared the template activity of the actin gene with that of the fibroin gene in vitro. For this purpose, the truncate DNA fragment having the actin promoter was transcribed together with that having the fibroin promoter by varying the molar ratios of the two templates in the same reaction mixture. Figure 2 shows the autoradiographic patterns of labeled RNA transcripts. In this system, when transcription starts from the promoters of the two genes, the sizes of the expected runoff transcripts will be approximately 390 and 300 bases long for the truncate templates from the actin and fibroin genes, respectively (Figure 2A). The efficiency of faithful transcription initiation of the two genes was compared at the molar ratios of 1:1, 4:1, 10:1, 20:1, and 50:1 between the actin and fibroin truncate templates (Figure 2B). The band intensity was densitometrically measured and then quantitatively compared after corrections were made for the molar ratio of the templates and the content of UMP in the transcripts. Even at the molar ratio of 50:1, the intensity of the 390-base RNA band of the actin gene transcript was still weaker than that of the 300-base transcript of the fibroin gene. Thus, in the in vitro system using the HeLa cell extract, the promoter activity of the actin 5 gene was 100–200 times weaker than that of the fibroin gene.

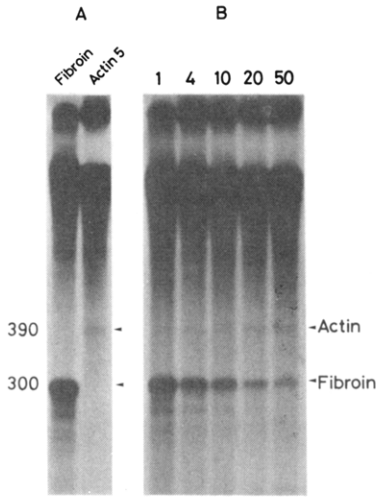

FIGURE 2: Comparison of the efficiency of in vitro transcription of actin and fibroin genes by the truncation assay using HeLa cell extract. The 3.1-kbp *Ava*II/*Hae*III fragment from pDd actin 5 or the 4.9-kbp *Sal*I-digested pFb200 3′Δ(+10) (A) or both (B) were added at 40 μg/mL to the reaction mixture (25 μL) containing 10 μL of HeLa cell extract (13 mg of protein/mL). In the experiments in which two templates were simultaneously assayed in one test tube (B), their molar ratio varied without changing the final DNA concentration. After the reaction mixture had been incubated for 60 min at 30 °C, the labeled RNA transcript was extracted, electrophoresed on 5% polyacrylamide–7 M urea gels, and autoradiographed. Numbers at the tops of the lanes (B) indicate the molar ratio of the actin gene to the fibroin gene.

Changes in the in Vitro Efficiency of Faithful Transcription Initiation of the Chimeric Genes. To determine the signal sequence affecting the in vitro efficiency of faithful transcription of the actin gene, we constructed a variety of the chimeric genes from the actin and fibroin genes and compared their transcription efficiencies in the HeLa cell extract. The chimeric genes used were largely divided into two groups. One

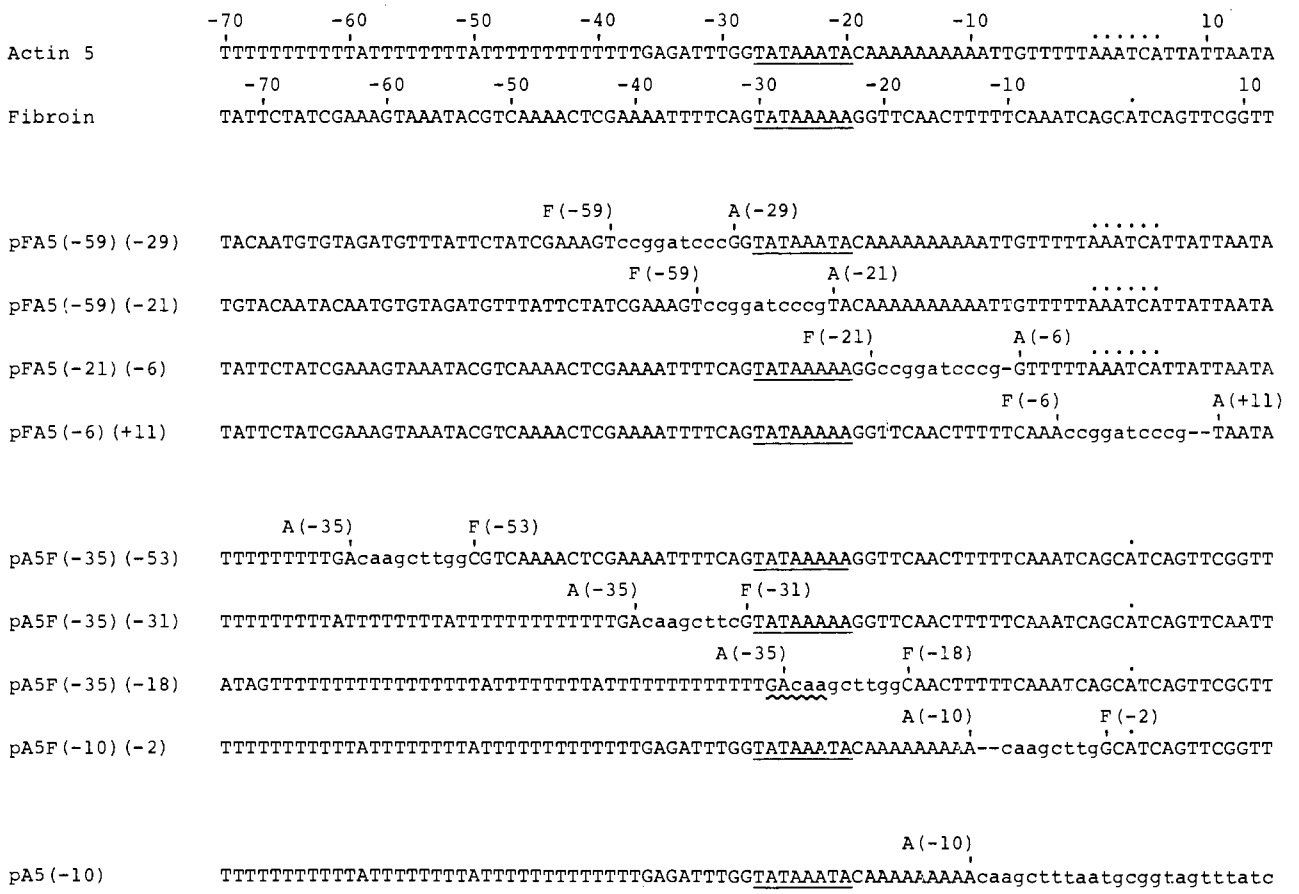

FIGURE 3: Nucleotide sequences of the regions around the TATA box and the initiation site of the deletion and chimeric genes. The TATA box is underlined and the TATA boxlike sequence is indicated by a wavy line. The transcription initiation sites of the fibroin and actin genes are shown by dots. The 5′-terminus of the primary transcript of the actin 5 gene was mapped by S1 nuclease analysis on several nucleotides of the region around 40–45 nucleotides upstream from the first nucleotide of the translation initiation codon (McKeown & Firtel, 1981; Takiya et al., 1984). Since it is unclear whether the above finding indicates multiple transcription initiation sites of the actin 5 gene or artifacts due to nibbling by S1 nuclease treatment, we tentatively assigned +1 to the adenine residue 44 nucleotides upstream from the first nucleotide of the translation initiation codon. The deleted portions between the TATA box and the transcription initiation site are hyphenated. Nucleotide sequences of the synthetic linkers and the vector DNA are denoted by small letters.

group was the pA5F series in which the downstream sequence of the actin 5 gene is replaced by the corresponding sequence of the fibroin gene. The other was the pFA5 series in which the relationship for the recombination between the two genes is the reverse of that of the pA5F; the upstream sequence of the actin gene is replaced by the upstream sequence of the fibroin DNA. Figure 3 shows the nucleotide sequences near the joining region of some representative chimeric genes.

In experiments using pFA5(−21)(−6) in which the upstream region including the actin TATA box was replaced by the corresponding one of the fibroin gene via a synthetic linker, the truncate DNA fragments prepared from the above recombinant plasmid with *Pst*I and *Hin*cII gave a transcript about 820 and 520 bases long, respectively. The size of the transcripts was as expected when transcription started from the promoter of the chimeric genes (Figure 4, lanes 1 and 3). Use of truncate templates prepared with *Xho*I and *Sma*I from pA5F(−10)(−2), which is a reciprocal chimeric gene of pFA5(−21)(−6), gave transcripts 520 and 190 bases long, which were expected for the initiation from the chimeric gene promoter (Figure 4, lanes 5 and 7). The RNA bands corresponding to these transcripts disappeared when the truncation assay was carried out in the presence of a low concentration (1 μg/mL) of α-amanitin (Figure 4, lanes 2, 4, 6, and 8). These results suggest that transcription of the chimeric genes was started almost accurately from the transcription initiation region by RNA polymerase II of the HeLa cell. The difference in the transcription efficiency between these two chimeric genes will be described below in more detail.

To assess the effect of replacing the promoter region of the actin gene with that of the fibroin gene, the recombinant plasmid pA5 3′Δ(+15) containing the sequence from −211 to +15 of the actin 5 gene was recloned into pBR322 and then used instead of the actin 5 gene. When the pA5 3′Δ(+15) DNA was cut with *Hin*cII and used as a template, it gave a transcript about 520 bases long, which was expected for the initiation from the actin 5 gene promoter (Figure 5, lane 2). The efficiency of the faithful transcription initiation from the actin promoter on the pA5 3′Δ(+15)e was somewhat higher than that on the 3.1 kbp *Ava*II/*Hae*III (+387) DNA fragment from the pDd actin 5 (cf. Figure 6A). This result was consistent with that of Grosschedl & Birnstiel (1982), who reported that the DNA fragment produced by truncation at various sites of the plasmid DNA influenced the efficiency of specific transcription on the truncate DNA fragment. The efficiency of specific transcription of the pA5 3′Δ(+15) was still very low relative to that of the mutant gene pFb200 3′Δ(+10) having the fibroin promoter, just as observed in the comparison of the in vitro transcription efficiency of the two natural genes (see Figure 9). However, with the pA5F-(−10)(−2) DNA, the efficiency of faithful transcription of the chimeric gene increased to several times that of the pA5 3′Δ(+15) (Figure 5, lanes 1 and 2), suggesting that the sequence in the transcription initiation region of the fibroin gene effectively promotes faithful transcription of the chimeric gene in vitro. When the truncation assay was performed with the

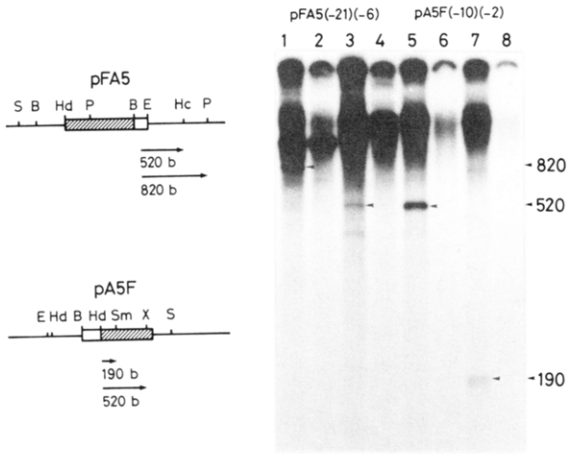

FIGURE 4: In vitro transcription of the chimeric genes joined between the TATA box and the transcription initiation site of the actin and fibroin genes. The templates were prepared from the pFA5(−21)(−6) DNA after treatment with *Pst*I or *Hin*cII and from the pA5F(−10)(−2) DNA after treatment with *Xho*I or *Sma*I. They were added to the reaction mixture containing HeLa cell extract and then incubated for 60 min at 30 °C in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of 1 μg/mL α-amanitin. RNA transcripts were extracted, electrophoresed on a 5% polyacrylamide–7 M urea gel, and autoradiographed. The structure of the chimeric genes and the expected size of transcripts are shown in the left half of the figure. Relevant restriction sites are shown by using the following abbreviations: Hc, *Hin*cII; B, *Bam*HI; Hd, *Hin*dIII; P, *Pst*I; E, *Eco*RI; Sm, *Sma*I; S, *Sal*I; X, *Xho*I. Open and hatched boxes indicate the actin and fibroin genes, respectively. Lanes 1 and 2, *Pst*I-digested pFA5(−21)(−6); lanes 3 and 4, *Hin*cII-digested pFA5(−21)(−6); lanes 5 and 6, *Xho*I-digested pA5F(−10)(−2); lanes 7 and 8, *Sma*I-digested pA5F(−10)(−2). In this figure, faint RNA bands other than the accurate transcripts can be seen. Though transcription start points of these RNAs were not examined, these RNAs seemed to be transcribed independently of transcription efficiency of the actin or fibroin promoter, because the intensity of the bands did not change, even when transcription efficiency of the corresponding promoter was altered (see Figure 6).

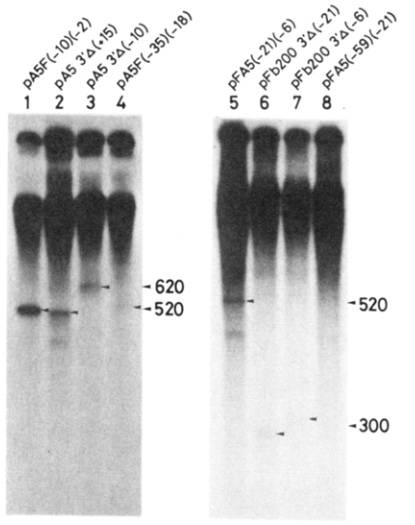

FIGURE 5: In vitro transcription of the chimeric genes and the deletion mutant genes deficient in the TATA box or the transcription initiation site. The concentration of DNA templates in the assay mixture was 40 μg/mL. Other conditions for the truncation assay were the same as those in Figure 2. The structures of the templates are schematically shown in Figure 9. Lane 1, *Xho*I-digested pA5F(−10)(−2); lane 2, *Hin*cII-digested pA5 3′Δ(+15); lane 3, *Sal*I-digested pA5 3′Δ(−10); lane 4, *Xho*I-digested pA5F(−35)(−18); lane 5, *Hin*cII-digested pFA5(−21)(−6); lane 6, *Sal*I-digested pFb200 3′Δ(−21); lane 7, *Sal*I-digested pFb200 3′Δ(−6); lane 8, *Hin*cII-digested pFA5-(−59)(−21).

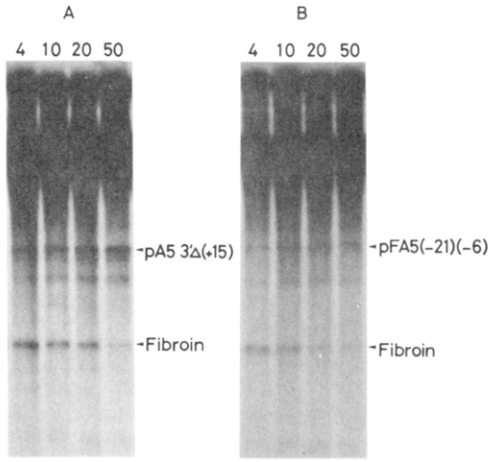

FIGURE 6: Comparison of the in vitro efficiency of specific transcription initiation of the actin deletion mutant pA5 3′Δ(+15) and the chimeric gene pFA5(−21)(−6). Templates were prepared from the pA5 3′Δ-(+15) DNA after digestion with *Hin*cII (A) and from the pFA5-(−21)(−6) DNA after digestion with *Xho*I (B). They were added to the reaction mixture and then incubated for 60 min at 30 °C together with the *Sal*I-digested pFb200 3′Δ(+10) DNA of which the template activity was an internal reference. The total concentration of the templates was adjusted at 40 μg/mL. The molar ratio of the template pA5 3′Δ(+15) or pFA5(−21)(−6) to pFb 200 3′Δ(+10) is indicated at the top of each lane. RNA transcripts were analyzed as described in Figure 2.

template from the chimeric gene pA5F(−35)(−18) that had neither the actin TATA box nor the fibroin one but the sequence of the *Hin*dIII linker in the region corresponding to the TATA box, we observed an RNA band of extremely low intensity and of the expected size for faithful transcription from the chimeric gene promoter (Figure 5, lane 4). The low specific transcription of this chimeric gene may be due to the artificial construction of a sequence resembling the TATA box at the joint region of the actin and fibroin genes [see Figure 3; see also Tsujimoto et al. (1981)].

In the truncation assay of the chimeric genes of the pFA5 series, pFA5(−21)(−6) was transcribed from the transcription start site of the actin 5 gene as efficiently as the pA5 3′Δ(+15) (Figures 6 and 9), suggesting that the sequence upstream from the transcription initiation site of the fibroin gene does not affect the efficiency of transcription from the actin transcription initiation site of the chimeric gene. In the in vitro transcription of the chimeric gene pFA5(−59)(−21) which has neither the actin TATA box nor the fibroin one, no transcription started from the actin transcription start site (Figure 5, lane 8). In addition, when the 3′-deletion mutant (having the TATA box) of the fibroin gene, which lacks the sequence downstream from position −21 or −6 but is complemented by the vector sequence, was transcribed as a control, we detected a very faint RNA band corresponding to the transcript of the expected size when transcription starts from the point comparable with the in vivo initiation site of the fibroin gene (Figure 5, lanes 6 and 7). A similar result was obtained in the truncation assay of the 3′-deletion mutant (having the TATA box), pA5 3′Δ(−10), of the actin gene (Figure 5, lane 3). Our results suggest that the TATA box of the actin gene is indispensable for transcription of this gene in vitro, as demonstrated with the fibroin gene or other eukaryotic class II genes. Also, the nucleotide sequence in the transcription initiation region of the actin or fibroin gene seems to strongly influence faithful and efficient initiation of transcription of these genes.

In Vitro Transcription of Chimeric Genes of Other Types. Here we investigated the effect of the upstream sequence of the TATA box of actin and fibroin genes on the efficiency of

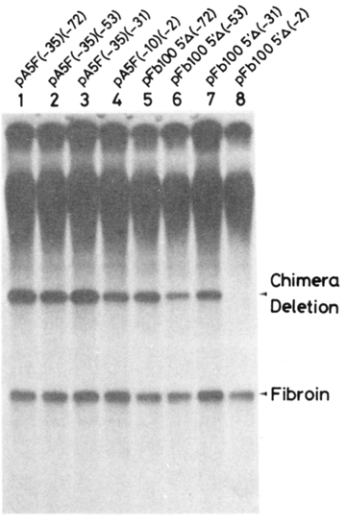

FIGURE 7: Comparison of the efficiency of specific transcription between the chimeric genes of the pA5F series and the corresponding fibroin deletion mutant gene in vitro. Templates were prepared from the pA5F DNA after cleavage with *Xho*I (see Figure 4) and incubated in the assay mixture for 60 min at 30 °C together with *Sal*I-digested pFb200 3′Δ(+10) DNA at a molar ratio of the template of 1:1. The final template concentration was adjusted to 40 μg/mL. RNA transcripts were analyzed as described in Figure 2. The structure of the chimeric gene is schematically shown in Figure 9. Lane 1, pAF(−35)(−72); lane 2, pA5F(−35)(−53); lane 3, pA5F(−35)(−10); lane 4, pA5F(−10)(−2); lane 5, pFb100 5′Δ(−72); lane 6, pFb100 5′Δ(−53); lane 7, pFb100 5′Δ(−31); lane 8, pFb100 5′Δ(−2).

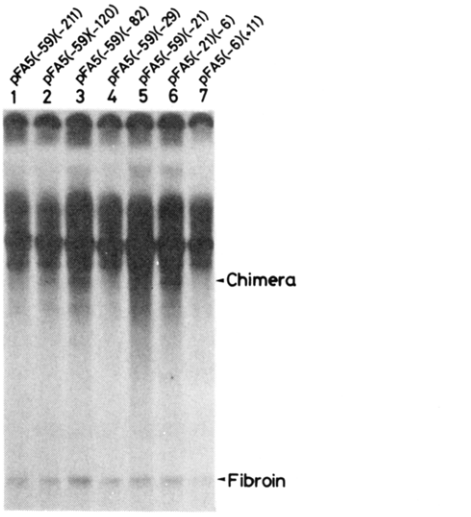

FIGURE 8: Comparison of the efficiency of specific transcription of chimeric genes of the pFA5 series in vitro. Templates were obtained from the pFA5 DNA after digestion with *Pst*I (see Figure 4) and incubated in the assay mixture for 60 min at 30 °C together with *Sal*I-digested pFb200 3′Δ(+10) DNA at a molar ratio of the template of 4:1. The final template concentration was adjusted at 40 μg/mL. RNA transcripts were analyzed as described in Figure 2. The structure of the chimeric gene is schematically shown in Figure 9. Lane 1, pFA5(−59)(−211); lane 2, pFA5(−59)(−120); lane 3, pFA5(−59)(−82); lane 4, pFA5(−59)(−29); lane 5, pFA5(−59)(−21); lane 6, pFA5-(−21)(−6); lane 7, pFA5(−6)(+11).

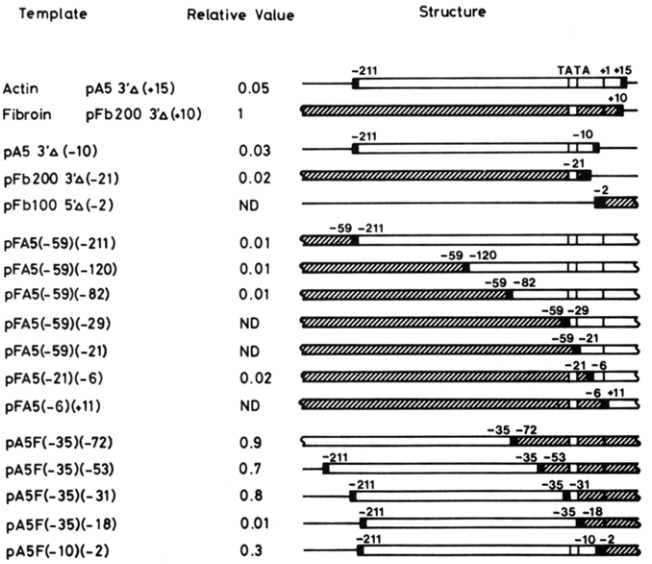

FIGURE 9: Quantitative comparison of the in vitro efficiency of specific transcription initiation of artificially constructed deletion and chimeric genes. The positional relation of the presumptive promoter region in the genes used here is schematically shown in the right half of the figure. Open squares represent the TATA box, and the other symbols are the same as those described in Figure 1. Details for the calculation of the relative value of the transcription efficiency are described under Materials and Methods. ND means that specific transcription was not detected.

their transcription. For this purpose, we constructed chimeric genes in which regions including both the TATA box and the transcription initiation site of the actin and fibroin genes were heterologously partnered. In serial experiments to assay the in vitro transcription of the chimeric genes, the truncate DNA fragment from the fibroin gene was added to all the assay mixtures as the internal reference to measure the transcription efficiency of various chimeric genes. Figure 7 shows that, in the chimeric genes of the pA5F series, the genes in which the TATA box and the transcription initiation site had originated from those of the fibroin gene were as efficiently transcribed as the natural fibroin gene, regardless of the joint sites of the two genes (compare lanes 1–3 with lanes 5–7). These results apparently suggest that the unusual AT-rich sequence upstream from the actin TATA box did not affect the efficiency of transcription initiation from the fibroin transcription start point in the chimeric genes in vitro. In agreement with the result reported by Tsujimoto et al. (1981), the 5′-deletion mutant of the fibroin gene which lacks the sequence upstream from position −2 was little transcribed in vitro from the in vivo initiation point (Figure 7, lane 8). However, when the deficient sequence in this fibroin deletion mutant was complemented by the corresponding upstream sequence of the actin 5 gene [corresponding to pA5F(−10)(−2)], transcription from the fibroin transcription initiation point was recovered and its efficiency increased to about one-third that of the natural fibroin gene (Figure 7, lane 4, and Figure 9). Therefore, the upstream sequence of the transcription initiation region of the actin 5 gene is not likely to be the main cause of the inefficiency of faithful transcription of this gene in vitro.

Figure 8 shows the results of the truncation assay of the chimeric genes of the pFA5 series. The chimeric genes, pFA5(−59)(−211), pFA5(−59)(−120), and pFA5(−59)(−82), were all transcribed to the same extent as the actin 5 gene (Figure 8, lanes 1–3; Figure 9). A similar result was obtained in the experiment with the pFA5(−21)(−6) gene which was constructed by ligation of the upstream sequence including the TATA box of the fibroin gene and the downstream sequence including the transcription initiation region of the actin gene [Figures 6B, 8 (lane 6), and 9]. This finding suggests that the upstream sequence including the fibroin TATA box exerts no positive effect on the efficiency of transcription from the actin transcription start site in the chimeric gene in vitro. When we tested in vitro transcription of the pFA5(−59)(−29) gene which had been constructed by joining the downstream sequence including the TATA box of the actin 5 gene and the upstream sequence close to the TATA box of the fibroin gene,

no faithful initiation of transcription from the actin transcription start region was observed (Figure 8, lane 4). This seemingly inconsistent phenomenon can be reasonably interpreted if it is assumed that the signal sequences to promote faithful transcription of the actin gene are localized near 5′ to the actin TATA box. The pFA5(−59)(−29) gene might have been deficient of the signal sequences. In agreement with the result of the truncation assay of the pA5F(−35)(−18) gene (see Figure 5, lane 4), no faithful transcription initiation was observed in the experiment with the pFA5(−59)(−21) which has neither the actin TATA box nor the fibroin one (Figure 8, lane 5), suggesting that the TATA box is essential for transcription of eukaryotic class II genes in vitro as pointed out by many investigators. We also did not observe any faithful transcription in the in vitro transcription test of the pFA5(−6)(+11) in which the transcription start region had been replaced by the *Bam*HI linker sequence (Figure 8, lane 7). This result strongly supports the idea that the nucleotide sequence in the transcription initiation region is indispensable for faithful and efficient initiation of transcription of the class II genes in vitro.

Discussion

Our study showed that the inefficient initiation of in vitro transcription of *D. discoideum* actin 5 gene in HeLa cell extract may be due to inadequacy of the structure of the transcription initiation site of this gene. In the pFA5 series, the chimeric genes having the transcription initiation site of the actin gene were transcribed with a very low efficiency, whereas the chimeric genes of the pA5F series, which have the initiation site of the fibroin gene, were transcribed with a relatively high efficiency. It was also evident that the actin and fibroin genes deprived of their transcription initiation regions were little transcribed from their promoters. These results strongly suggest that the sequence of the transcription initiation region is important for promoting the in vitro transcription of actin and fibroin genes.

In addition, the fact that the pA5F chimeric genes, which have the long AT-rich sequence derived from the actin gene in the upstream region of the TATA box, were relatively well transcribed implies that this AT-rich sequence has no negative effect on transcription from the promoter of the actin gene in vitro and also suggests that the upstream sequence of the actin TATA box is not the cause of inefficient initiation of transcription of the actin 5 gene in vitro. This conclusion agrees with the evidence that, in several class II genes, the upstream sequence of the TATA box is not always essential for raising the fidelity and efficiency of transcription of the genes in the in vitro system containing HeLa cell extract (Wasylyk et al., 1980; Grosveld et al., 1981; Tsujimoto et al., 1981). However, some effective sequences for in vivo and in vitro transcription have been found in the region around 200 bp upstream from the initiation site of many class II genes such as yeast HIS4 (Denahue et al., 1983), human interferon α (Weidle & Weissmann, 1983), rabbit β-globin (Dierks et al., 1983), sea urchin histone H2A (Grosschedl & Birnstiel, 1983), and fibroin genes (Tsuda & Suzuki, 1981, 1983). Therefore, although further experiments will be necessary to reach a clear conclusion as to the significance of the upstream sequence of the actin TATA box, the transcription factor(s) which may be contained in the HeLa cell extracts is (are) probably not readily available for the actin gene promoter as it acts efficiently on other genes due to its specificity for species, tissues, or genes. In fact, Dynan & Tijian (1983a,b) have isolated from HeLa cell extract a fraction for specific transcription of SV40 early gene.

Studies on the role of the transcription initiation site in class II genes have yielded inconsistent results. Removal of the transcription initiation site of conalbumin (Corden et al., 1980), fibroin (Tsujimoto, et al., 1981), and mouse α-globin genes (Talkington & Leder, 1982) caused a marked decrease in the level of faithful transcription of these genes in HeLa cell extract. Similar results were also obtained with Ad2 major late (Corden et al., 1980) and Ad2 early III genes (Lee et al., 1982). An important role of the initiation site in transcription of the fibroin gene has been suggested by Suzuki and his co-workers in the homologous transcription system containing the cell extract prepared from *Bombyx mori* silk gland cells (Tsuda & Suzuki, 1981, 1983) as well as in the heterologous system using HeLa cell extract (Tsujimoto et al., 1981). Recently confirmative evidence has been obtained from in vivo experiments with transformed COS cells as well (Tokunaga et al., 1984). Talkington & Leder (1982) have shown that transcription of a pseudogene of mouse α-globin gene could be rescued by replacing the transcription initiation site with that of the gene normally expressed in vivo. However, deletion or substitution of the nucleotide sequence of the transcription initiation site of rabbit β-globin (Grosveld et al., 1981; Grosveld et al., 1982; Dierks et al., 1983), SV40 early (Benoist & Chambon, 1981; Myers et al., 1981), and HSV thymidine kinase genes (McKnight et al., 1981; McKnight & Kingsbury, 1982) does not appear to affect the efficiency of faithful transcription initiation of these genes both in vivo and in vitro. Grosschedl & Birnstiel (1980, 1982) reported that, in sea urchin histone H2A gene, the transcription efficiency of the gene deleting the initiation site did not differ from that of the wild-type gene in vitro but markedly decreased in vivo. Recently, Davison et al. (1983) found that the DNA template having the TATA box but not the transcription initiation site could not form the stable preinitiation complex for transcription. Our unpublished data have suggested that the in vitro efficiency of faithful initiation of transcription of the chimeric gene build from the actin 6 and fibroin genes greatly depends on the distance from the TATA box to the transcription initiation point (K. Takahashi, et al., unpublished results). Considering our present results and unpublished finding, we concluded that the transcription initiation site should be regarded as a part of the promoter, although its significance for the transcription somewhat differs from gene to gene. The structural difference of the transcription initiation site is assumed to be reflected by the difference in the transcription efficiency of the respective gene in vivo.

There may be the most suitable sequence for a structure of the transcription initiation region, because in many cases deletions or substitutions of nucleotides in the transcription initiation region can cause a decrease in the faithful and efficient transcription of the class II genes [see, for example, Tokunaga et al. (1984)]. However, no universally accepted sequence has yet been found except that transcription of almost all the class II genes starts from the adenine residue in the pyrimidine-rich sequence downstream around 30 bp from the TATA box (Corden et al., 1980; Sasson-Corsi et al., 1981). Therefore, in addition to the enhancer element which has recently become important as one of the modulators in transcription, further studies on the relation between the structure and function of the transcription initiation region are needed.

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References

Benoist, C., & Chambon, P. (1981) *Nature* (*London*) *290*, 304–310.

Corden, J., Wasylyk, B., Buchwalder, A., Sasson-Corsi, P., Kédinger, C., & Chambon, P. (1980) *Science* (*Washington D.C.*) *209*, 1406–1414.

Davison, B. J., Egly, J.-M., Mulvihill, E. R., & Chambon, P. (1983) *Nature* (*London*) *301*, 680–686.

Denahue, T. F., Daves, R. S., Lucchini, G., & Fink, G. R. (1983) *Cell* (*Cambridge, Mass.*) *32*, 89–98.

Dierks, P., van Oyen, A., Cochran, M. D., Dobkin, C., Reiser, J., & Weissmann, C. (1983) *Cell* (*Cambridge, Mass.*) *32*, 695–706.

Dynan, W. S., & Tjian, R. (1983a) *Cell* (*Cambridge, Mass.*) *32*, 669–680.

Dynan, W. S., & Tjian, R. (1983b) *Cell* (*Cambridge, Mass.*) *35*, 79–87.

Firtel, R. A., Timm, R., Kimmel, A. R., & McKeown, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6206–6210.

Firtel, R. A., McKeown, M., Poole, S., Kimmel, A. R., Brandis, J., & Rowekamp, W. (1981) in *Genetic Engineering* (Setlow, J. K., & Hollaender, A., Eds.) Vol. 3, pp 265–318, Plenum Press, New York.

Grosschedl, R., & Birnstiel, M. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1432–1436.

Grosschedl, R., & Birnstiel, M. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 297–301.

Grosveld, G. C., Shewmaker, C. K., Jat, P., & Flavell, R. A. (1981) *Cell* (*Cambridge, Mass.*) *25*, 215–226.

Grosveld, G. C., de Boer, E., Shewmaker, C. K., & Flavell, R. A. (1982) *Nature* (*London*) *295*, 120–126.

Hen, R., Sasson-Corsi, P., Corden, J., Gaub, M. P., & Chambon, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7132–7136.

Hirose, S., Takeuchi, K., & Suzuki, Y. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7258–7262.

Kindle, K. L., & Firtel, R. A. (1978) *Cell* (*Cambridge, Mass.*) *15*, 763–778.

Lee, D. C., Roeder, R. G., & Wold, W. S. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 41–45.

Manley, J. L., Fire, A., Sharp, P. A., & Gefter, M. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3855–3859.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–550.

McKeown, M., & Firtel, R. A. (1981) *Cell* (*Cambridge, Mass.*) *24*, 799–807.

McKnight, S. L., & Kingsbury, R. (1982) *Science* (*Washington D.C.*) *217*, 316–324.

McKnight, S. L., Gavis, E. R., Kingsbury, R., & Axel, R. (1981) *Cell* (*Cambridge, Mass.*) *25*, 385–398.

Melon, P., Parker, V., Gluzman, Y., & Maniatis, T. (1981) *Cell* (*Cambridge, Mass.*) *27*, 279–288.

Myers, R. M., Rio, D. C., Robbins, A. K., & Tjian, R. (1981) *Cell* (*Cambridge, Mass.*) *25*, 373–384.

Sasson-Corsi, P., Corden, J., Kédinger, C., & Chambon, P. (1981) *Nucleic Acids Res. 9*, 3941–3958.

Takiya, S., Tabata, T., Iwabuchi, M., Hirose, S., & Suzuki, Y. (1984) *J. Biochem.* (*Tokyo*) *95*, 1367–1377.

Talkington, C. A., & Leder, P. (1982) *Nature* (*London*) *298*, 192–195.

Tokunaga, K., Hirose, S., & Suzuki, Y. (1984) *Nucleic Acids Res. 12*, 1543–1558.

Tsuda, M., & Suzuki, Y. (1981) *Cell* (*Cambridge, Mass.*) *27*, 175–182.

Tsuda, M., & Suzuki, Y. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7442–7446.

Tsujimoto, Y., Hirose, S., Tsuda, M., & Suzuki, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4838–4842.

Wasylyk, B., Kédinger, C., Cordon, J., Brison, O., & Chambon, P. (1980) *Nature* (*London*) *285*, 367–373.

Weidle, V., & Weissmann, C. (1983) *Nature* (*London*) *303*, 442–446.