***In vitro* accurate initiation of transcription on the adenovirus type 2 IVa2 gene which does not contain a TATA box**

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ABSTRACT

In vitro initiation of transcription has been studied on the IVa2 gene of the adenovirus type 2 (Ad2) which is expressed at intermediate time after infection. This gene does not contain a TATA box around 30 bp upstream from the cap site. By analyses of the in vitro runoff products by size and by single strand nuclease protection mapping, it was concluded that the in vitro initiation occurred accurately at two in vivo cap sites with low but detectable efficiency. By using cloned IVa2 DNA, which is free from the major late promoter, it was suggested that the low efficiency of the in vitro initiation on the IVa2 gene may not be due to close location of the two genes but may rather reflect the efficiency of the promoter itself. The cell-free extracts from both uninfected and infected HeLa cells supported the accurate initiation of transcription in vitro with almost the same efficiency. In addition, the cell free extract directed an artificial initiation of transcription in vitro by recognizing a TATA box located 140 bp upstream from the cap sites.

INTRODUCTION

Recent studies on the transcriptional control of gene expression in eukaryotes have greatly progressed by introducing an in vitro transcription system reconstituted with a cell-free extract and purified DNA (1-4). In these systems so far studied, there is much evidence that the initiation of transcription is carried out faithfully at the in vivo initiation sites of any classes of genes (I-rRNA gene, II-mRNA and some snRNA genes, and III-tRNA, 5sRNA, Ad2 associated RNA and some snRNA genes) (1-9).

It is well known that most of class II genes, transcribed with RNA polymerase II, contain an A+T rich sequence (the Goldberg-Hogness or TATA box) about 30 bp upstream from the in vivo cap site. An application of the deletion mutant genes in vitro and in vivo has revealed the TATA box to play an important role for transcription to occur at the cap site (for review, see ref. 10). However, there are a few genes such as early EII and IVa2 genes of Ad2, and late genes of papovavirus which do not contain the TATA box at the site generally observed (11-13). This suggests that some other type of promoter than the TATA box might be present in those genes, and/or that the TATA

box might not always be essential as the promoter.

Recently it has been shown that the multiple factors are required for an accurate initiation of transcription in vitro with RNA polymerase II (14). Although whether these factors are common to any genes containing the TATA box remains to be studied, one could ask whether different factors are involved in the initiation of transcription on the genes not containing the TATA box.

In an attempt to elucidate the promoter sequence and the transcription factors required for specific initiation of transcription on the non-TATA box genes, I first examined whether the IVa2 gene of Ad2 could be transcribed accurately from the in vivo cap site in the in vitro transcription system.

MATERIALS AND METHODS

Cell culture and infection of cells with virus. HeLa cells were maintained in suspension cultures in Joklik's modified minimal essential medium (GIBCO) supplemented with 5% horse serum (GIBCO). Ad2 was kindly provided by Dr. T. Yamashita, Kyoritsu University, Japan. HeLa cells in mid-log phase growth were concentrated to 1×10^7 /ml, and 200 pfu per cell of Ad2 were added. After 60 min of incubation at 37°C, cells were diluted to 4×10^5 /ml and grown at 37°C.

Preparation of cell-free extract. The cell-free extract was prepared from uninfected and infected HeLa cells according to Weil et al. (2) with some modifications as described below. The cell homogenate was added to one-tenth volume of 10 x buffer (0.3M HEPES pH 7.9, 1.4M KCl and 30mM MgCl₂) and was centrifuged at 2000 x g for 10 min. The supernatant was further centrifuged at 100,000 x g for 60 min. The nuclear pellet obtained by low speed centrifugation of the cell homogenate was resuspended in a buffer (20mM HEPES pH 7.9, 25% glycerol, 5mM MgCl₂, 0.2mM EDTA, 0.4M KCl and 0.5mM DTT). After being placed on ice for 15 min, the suspension was centrifuged at 100,000 x g for 60 min. The resulting supernatant from both cytoplasmic and nuclear fractions were mixed together and dialyzed against a buffer (20mM HEPES pH 7.9, 25% glycerol, 0.2mM EDTA, 0.1M KCl and 0.5mM DTT) for several hours. The dialysate was centrifuged at 10,000 x g for 15 min and the supernatant was stored at -80°C until use.

Cloned DNA. pSmaF plasmid was obtained from Drs. J. Segall and S. Hirose, separately. pAdIVa2 plasmid was constructed as follows; the HpaII fragment of the SmaI-F DNA, which contains 5'-end flanking region of the IVa2 gene but not the promoter region of the major late gene, was isolated and ligated to the BamHI site of pBR322. pHindC plasmid was constructed by ligating the HindIII-

C fragment of Ad2 DNA to the HindIII site of pBR322.

In vitro transcription and analysis of products. The standard reaction mixture contains in 50 μ l; 100 μ Ci/ml of α -³²P-GTP, 30 μ M each of unlabeled ribonucleotide triphosphates, 15 μ M HEPES pH 7.9, 40mM KCl, 6mM MgCl₂, 30 μ l of cell extract, 1 μ g/ml of isolated DNA fragment and 12 μ g/ml of poly[d(A-T)]-[d(A-T)] (15) or 12 μ g/ml of plasmid DNA, and 30 units of HeLa cell RNA polymerase II. The reaction was carried out at 30°C for 60 min. The purification of the RNAs was as described in Weil *et al.* (3). The purified RNAs were analyzed on a 4% or 12% polyacrylamide gel after treatment with glyoxal at 50°C for 60 min, or analyzed directly on a 4% polyacrylamide/urea gel.

Single strand nuclease mapping of the in vitro RNA. RNA was synthesized in 3 fold standard reaction mixture without labeled nucleotide on the purified 1.1 kb PvuII DNA fragment and was treated with 100 μ g/ml of RNase-free DNase at 37°C for 30 min after purifying (for one protection mapping). The purified XhoI/PvuII DNA fragment was labeled at 5'-ends and the coding strand was isolated on a 4% polyacrylamide gel. RNA and the coding strand DNA were coprecipitated with ethanol and dissolved in 15 μ l of 50% formamide-20mM PIPES pH 6.4-0.4M NaCl-1mM EDTA. Hybridization was performed at 37°C for 15 hours after heating at 85°C for 5 min. The hybridization solution was diluted into 150 μ l of cold P1 buffer (16) and incubated with nuclease P1 at 37°C for 60 min followed by ethanol precipitation in the presence of carrier tRNA. The sample was run on a 12% polyacrylamide/urea sequencing gel along with chemically cleaved coding strand DNA used as probe for hybridization (17). For comparison, total cytoplasmic RNA isolated from Ad2 infected HeLa cells was subjected to the same procedure.

Radioisotopes and enzymes. γ -³²P-ATP (~3000 Ci/mmol) and α -³²P-GTP (~400 Ci/mmol) were purchased from Amersham. Restriction endonucleases were obtained from Takara Shuzo Co., Bethesda Research Laboratories, Inc. and New England Biolabs, Inc.. Nuclease P1 was a generous gift from Dr. M. Fujimoto (Yamasa Shoyu Co.). Bacterial alkaline phosphatase and DNase were purchased from Worthington Biochemical Corp.. The DNase was treated with sodium iodoacetate to remove contaminating RNase.

RESULTS

Runoff transcripts from the IVa2 gene region in vitro.

The IVa2 gene, expressed at intermediate time in lytic cycle of infection, is localized adjacent to the major late gene and transcribed from the λ strand of the Ad2 genome (Figure 1 and see refs. 18,19). Baker and Ziff have

Figure 1. (a) Position of IVa2 and major late (ML) transcription units on the Ad2 genome. Vertical lines between capital letters denote the SmaI sites. (b) Nucleotide sequences around the cap sites of the IVa2 and the major late genes (see ref. 12). Arrow heads indicate the cap sites. Two TATA boxes are also shown.

identified the two cap sites of this gene at 15.85 map units (m.u.), which are 210 and 212 bp to the left of the major late cap site (12). Thus the SmaI F fragment contains both the IVa2 and the major late genes promoter regions. When the pSmaF DNA truncated with SmaI and PstI was used as template in an *in vitro* transcription system, three distinct transcripts of ~1000, ~900 and ~560 nucleotides were obtained, which were completely inhibited with a low concentration of α -amanitin, indicating that these were RNA polymerase II products (Figure 2a, lane a and b). The band of ~560 nucleotides, which is the major late gene runoff transcript, disappeared when the pSmaF DNA truncated with PvuII was used as template, because the PvuII site was located 33 bp downstream from the major late cap site. However, the other two transcripts of ~1000 and ~900 nucleotides appeared again with this template (Figure 2a, lane c and d). As seen in Figure 2b, the PvuII site was very close to the PstI site on the IVa2 gene region. Thus it is easily expected that the transcripts from those two differently truncated pSmaF DNA had almost the identical sizes if the initiation of transcription was occurred around the *in vivo* cap sites. Furthermore, the isolated 1.1 kb PvuII DNA fragment gave the identical

Figure 2. Analysis of RNA transcripts synthesized *in vitro*. (a) RNA was synthesized, purified, glyoxylated, electrophoresed on 4% (lanes a-h) or, 12% (lanes i and j) polyacrylamide gels and autoradiographed. The cell extract from Ad2 infected cells was used in all assays. Lanes a and b: pSmaF DNA digested with SmaI and PstI. Lanes c and d: pSmaF DNA digested with PvuII. Lanes e and f: 1.1 kb PvuII fragment. Lanes g and h: 0.8 kb RsaI fragment. Lanes i and j: pHindC DNA digested with SstI. Lane M: mixture of three kinds of pBR322 DNA digests (pBR322 DNA digested with HinfI, HinfI-BamHI and HinfI-HindIII). In lanes b, d, f, h and j, incubation was performed in the presence of 1 μ g/ml of α -amanitin. (b) Restriction map of the SmaI-F fragment of Ad2 DNA. Arrow heads indicate the cap sites of the IVa2 and the major late genes. Arrows indicate the direction and the length (in nucleotides) of runoff transcripts expected. ∇ : SmaI, $\blacksquare$: PvuII, ∇ : RsaI, ∇ : PstI, $\uparrow$: SstI, $\uparrow$: XhoI.

transcripts of ~ 1000 and ~ 900 nucleotides in size (Figure 2a, lane e and f). Judging from the size of the transcripts, the runoff transcript of ~ 900 nucleotides appeared to be initiated around the *in vivo* cap sites. This was further confirmed by using the DNA truncated with different restriction endonucleases. As shown in Figure 2a, lane g-j, the truncation of DNA with RsaI

Figure 3. Analysis of RNA transcripts synthesized *in vitro*. (a) RNA was synthesized, purified and analyzed on 4% polyacrylamide/urea gels. The cell extract from the infected cells was used in all assays. Lane a: 1.1 kb PvuII fragment of pSmaF DNA. Lanes b and c: 2 µg/ml of EcoRI/SalI fragment of pAdIVa2 DNA. Lanes d and e: 10 µg/ml of the same DNA as in lanes b and c. In lanes c and e, incubation was performed in the presence of 1 µg/ml of α-amanitin. (b) Construction of pAdIVa2 plasmid. Positions of two TATA boxes, and the cap sites of the IVa2 and the major late genes were shown (upper). The HpaII fragment (280 bp) was inserted into the BamHI site of pBR322 DNA as depicted (lower). Thick line and thin line represent the insert and pBR322 DNA, respectively. Arrow indicates the direction and the length (in nucleotides) of runoff transcript expected.

or SstI gave, as expected, the transcript of ~600 or ~190 nucleotides, respectively. I thus concluded that the cell extract from HeLa cells infected with Ad2 could direct the specific initiation of transcription around the *in vivo* cap sites of the IVa2 gene.

Another transcript of ~1000 nucleotides observed with the pSmaF/SmaI-PstI or PvuII appeared again with the isolated 1.1 kb PvuII DNA fragment as template (Figure 2a, lane e, Figures 3 and 5). When variously truncated DNA was used as template, the length of this transcript varied accordingly but was consistently about 100 nucleotides longer than the transcript initiated from the *in vivo* cap sites (Figure 2a, lane g-j and Figure 3). As seen in Figure 1b, a typical TATA box is localized 140 bp upstream from the cap sites of the

IVa2 gene. Thus the about 100 nucleotides longer transcript is probably initiated by recognizing this TATA box as the promoter.

Efficiency of the initiation of transcription on the IVa2 promoter.

As seen in Figure 2a, lane a, the rate of specific initiation of transcription from the IVa2 promoter appeared to be very low (less than 10% of that of the major late promoter). Since the IVa2 and major late genes are closely located with their cap sites 210 bp apart (Figure 1b), the low rate of transcription on the IVa2 gene could result from this close location of the two genes. In order to examine this possibility, a recombinant plasmid designated pAdIVa2, which was free from the TATA box of the major late gene, was constructed (Figure 3b). When the isolated 950 bp EcoRI/SalI fragment of this plasmid was used as template, two distinct α -amanitin sensitive transcripts of ~ 470 and ~ 370 nucleotides were obtained (Figure 3a, lane b-e). By use of another truncated template, it was confirmed that these transcripts were initiated at the same sites as indicated earlier (data not shown). Compared with the intensity of the ~ 900 nucleotides band in lane a, that of ~ 370 nucleotides band which was initiated at the cap sites was not significantly enhanced by removal of the major late gene promoter. The ~ 470 nucleotides transcript, which was initiated by recognizing the TATA box, was also synthesized inefficiently, although the intensity of this band varied with different assays (see Figures 2, 3 and 5). These observations simply lead to the conclusion that the low rate of transcription of the IVa2 gene is not due to the close location of the major late promoter but due to the weakness of the promoter itself. However, possibility may still exist that there could be sequences, probably in the upstream region, which modulate the rate of initiation of this gene.

Single strand nuclease mapping of in vitro transcripts.

As shown in Figure 2, the cell extract can direct the specific initiation of transcription around the in vivo cap sites on the IVa2 gene. To more precisely locate the 5'-ends of the in vitro transcripts and compare them with the 5'-ends of the corresponding in vivo RNAs, single strand nuclease protection mapping analysis was carried out together with a DNA sequencing ladder. As shown in Figure 4, the in vitro transcripts gave two sets of protection bands as indicated with the arrow heads (lane b). The lower protection bands in lane b were similar to those with the in vivo RNAs in lane c, although each protection band with the in vivo RNAs was smaller by one nucleotide than that with the in vitro transcripts. This difference seemed to be due to an end trimming with nuclease P1, because the in vitro transcripts also

Figure 4. Localization of the 5'-ends of the in vitro transcripts and in vivo RNAs by single strand nuclease protection mapping with DNA sequencing ladder. The coding strand DNA was prepared from the labeled XhoI/PvuII fragment of the SmaI-F DNA and was hybridized with in vitro transcripts (lane b) or in vivo RNAs (lane c). 0.5 μ g/ml of nuclease P1 was used for digestion. The T+C cleaved DNA sequence ladder from same DNA fragment is shown in lane a. Arrow heads on the left side of lane a indicate the cap sites identified by Baker and Ziff (12). The numbered arrow heads in lane b indicate the position of protected fragment. Position 2 and 3 correspond to A and T, respectively.

gave identical set of protection bands when digested with a larger amount of nuclease (data not shown). As previously identified by Baker and Ziff (12), the IVa2 mRNA has two 5'-ends, U and A. Taking into account that the sequence ladder represents the coding strand, it can be deduced that bands 2 and 3 correspond to A and T, respectively, when correction of $1\frac{1}{2}$ nucleotides was made according to Sollner-Webb and Reeder (20). In addition to the protection bands corresponding to initiation of transcription at position +1 and +3, the in vitro transcripts gave another protection, band 1. On a 8% polyacrylamide/urea sequencing gel, this protection showed two bands which corresponded to T and C at position -110 and -111, respectively (data not shown). These positions were localized 29 and 30 bp downstream from the TATA box present in the 5'-end flanking region of the IVa2 gene (see Figure 1b), corroborating the results of in vitro runoff assays. However, with the in vivo RNAs no such protection was obtained. From these results, I concluded that the cell extract from the infected cells can direct the in vitro initiation of transcription precisely at the in vivo cap sites of the IVa2 gene and that the cell extract can direct an artificial initiation of transcription by recognizing an upstream TATA box as promoter.

Figure 5. Ability to direct the specific initiation of transcription with the cell extracts from mock infected and virus infected cells. RNA was synthesized and analyzed as described in Figure 2. Lane a: the cell extract from mock infected cells. Lane b: the cell extract from virus infected cells at 5 hours after infection. Lane c: the cell extract from virus infected cells at 15 hours after infection. The 1.1 kb PvuII fragment was used as template. Arrow heads indicate the IVa2 gene transcripts.

The ability of the cell extract from the uninfected HeLa cells

In the experiments shown above the cell extract from the virus infected cells was used in an in vitro transcription system. In an in vivo situation, the expression of the IVa2 gene is shown to be regulated during the course of virus infection (21). To investigate if the in vitro transcription system using the non-TATA box gene reflects a regulatory function observed in vivo, cell extracts were prepared from the mock-infected cells and from the cells in the early stage of infection and the ability to direct the specific initiation of transcription from the IVa2 promoter was examined. As shown in Figure 5, lane a-c, the cell extracts from any stage of cells could support the specific initiation of transcription on the isolated 1.1 kb PvuII DNA fragment with similar efficiency. Use of the fragment which is extended as far as the SmaI site (746 bp from the IVa2 cap sites) gave the same result as shown in Figure 5, thus excluding the possibility that the sequence flanked by the PvuII and SmaI sites have some regulatory function (data not shown)

DISCUSSION

In this report I have shown that the cell-free extract can direct an ac-

curate initiation of transcription on the IVa2 gene which, unlike most of class II genes, does not contain a TATA box around 30 bp upstream from the in vivo cap sites. This indicates that the in vitro initiation of this gene is apparently independent of any function of the TATA box. The rate of initiation of transcription on this gene was quite low, less than 10% of that on the major late gene. At present, it is not clear whether this low efficiency of initiation from the non-TATA box gene is due to the absence of the TATA box or to weakness of the promoter itself. However, it should be mentioned here that even the mutant gene deleting the TATA box is still active in vitro with an efficiency of $\sim 5\%$ of the wild type gene (22,23). This observation, coupled with the findings described in this paper, seems to lead to the following idea; the TATA box is essentially not required for in vitro initiation of transcription.

It is generally accepted that the TATA box plays an important role for transcription to initiate at the in vivo cap site. Also, the TATA box is absolutely required for maximal level of the in vitro initiation of transcription because of the significant decrease of the level of initiation caused by deleting this sequence (22-29). On the contrary, deletion of the TATA box does not significantly reduce the initiation of transcription in vivo, but does generally give transcripts with widely dispersed 5'-termini (30-34). Consequently, it has been proposed that a primary role of the TATA box is to specify a precise initiation site within a narrow area (30). This discrepancy observed in vitro and in vivo may be resolved if one assumes that the TATA box is not of primary but rather of secondary importance for initiation of transcription in vivo. Thus, the process occurring in vitro may reflect only part of a more complex in vivo process. It is probable that the TATA box constitutes a site of high affinity for RNA polymerase II and the transcription factor to bind and thereby precisely positions the initiation complex at the cap site.

Recently, Mathis et al. reported the accurate initiation of transcription in vitro at early cap site of Ad2 EII region which does not contain the TATA box (35). The rate of initiation of this gene, like the IVa2 gene, is also quite low. Although there seems to be no homology in the 5'-end flanking region between these non-TATA box genes, it is very important to identify their promoter sequences for understanding the nature of the class II gene promoter.

It has been shown that there are multiple factors involved in the in vitro initiation of transcription with RNA polymerase II (14). Although the exact function of each factor at the initiation step is yet to be clarified, it

is very important to ask whether the same set of factors are involved in the initiation of transcription on the non-TATA box genes. Along this line, experiments with fractionated extract are in progress.

In the present experiments, the IVa2 gene whose expression is regulated under the other early viral function could be transcribed with the cell extract from the uninfected cells. In the in vitro transcription systems so far studied, any genes are active even with the cell extract from cells which do not express them. This simply indicates that any positive regulatory factors are not absolutely required for the initiation of transcription in vitro on purified DNA template. Taking into consideration that the efficiency of the in vitro initiation is exceedingly low (0.002-0.02 transcripts per hour per template, see ref. 3), the initiation observed in in vitro reaction could reflect the background level in vivo. It is also possible that the cell-free extract prepared by the present method does not contain a factor which would modulate a level of initiation. Much evidence has been recently accumulated that an upstream region beyond 60 bp from the cap site possesses a crucial effect on a level of initiation of transcription in vivo (30, 32-34, 36-38). In this context, it is notable that this upstream region of some genes actually influences the in vitro initiation (29, 39).

Another possibility, and most likely, is that the in vitro transcription system reconstituted with purified DNA is not suitable for studying how an expression of gene is regulated. A functional region responsible for the regulation of the initiation of transcription may be covered with molecules such as histones or other proteins, which may repress the expression of the corresponding gene. As far as naked DNA being used as template, this repression remains released and the initiation of transcription can occur without regulatory factors. In this regard, one should again try to use chromatin as template in vitro coupled with the cell-free extract. Recently, Klang et al. showed that a viral transcription complex from Ad2 infected cells retained the ability to elongate the RNA chain initiated in vivo over distances greater than 10 kb and to carry out correct polyadenylation in vitro (40). Although whether RNA polymerase II can initiate, or reinitiate, in this transcription complex is not clear, it may be worth examining this system in the search for regulatory factors.

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