Expression of Circular Plasmids Which Contain Bacterial Chloramphenicol Acetyltransferase Gene Connected to the Promoter of Polypeptide IX Gene of Human Adenovirus Type 12 in Oocytes, Eggs and Embryos of Xenopus laevis

YUCANG FU, KENZO SATO, KEICHI HOSOKAWA
and KOICHIRO SHIOKAWA

Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, and
1Department of biochemistry, Kawasaki Medical College, Kurashiki 701-01, Japan

ABSTRACT—Polypeptide IX gene of adenovirus type 12 is unique in that it is expressed intermediately inbetween early and late genes, but the structure and function of its 5'-upstream promoter region have not been well characterized. In the present experiment, fertilized eggs as well as oocytes and unfertilized eggs of *Xenopus laevis* were injected with circular plasmid, pAd12.IXCAT, which contains bacterial chloramphenical acetyltransferase (CAT) gene fused to the promoter of polypeptide IX gene of adenovirus type 12, and the activity of this plasmid to promote CAT enzyme expression in *Xenopus* embryonic cells was examined. For comparison, pAd12.ElaCAT which contains the promoter of Ela protein of adenovirus type 12, pSV2CAT which contains SV40 early promoter, and pSV0CAT and PA10CAT3m which do not contain promoter were also tested. In the oocyte nucleus, all these circular plasmids were expressed similarly actively. In embryos and unfertilized eggs, however, while pAd12.ElaCAT and pSV2CAT were strongly expressed, level of the expression of pAd12.IXCAT was as low as those of pSV0CAT and PA10CAT3m. These results show that the promoter of polypeptide IX gene of adenovirus type 12 is very weak as compared with that of Ela protein.

INTRODUCTION

The polypeptide IX is associated with the group of nine hexons of adenovirus virion, and may play a cementing role in the assemblage of virus particle [1, 2]. Polypeptide IX is expressed intermediately inbetween the early and late phases of adenovirus infection, and the regulation of its synthesis appears to differ from that of other structural polypeptides of the virus particle [3]. Polypeptide IX is unique in that it is encoded by a relatively small mRNA of about 9S (ca. 485 b), and unlike other adenovirus mRNAs, the formation of polypeptide IX mRNA does not involve splicing [4]. To characterize the promoter function, Kruczek and Doerfler [5] isolated polypeptide IX gene from adenovirus type 12 genome, and after connecting its promoter to CAT gene, studied the effect of methylation on the promoter function. However, the expression of CAT enzyme activity from the fusion gene has not yet been studied in other eukaryotic cell system. In the present experiment, fertilized eggs as well as oocyte nuclei and unfertilized eggs of *Xenopus laevis* were injected with pAd12.IXCAT, a plasmid which contains the promoter of polypeptide IX of adenovirus type 12 [5] and the activity of the plasmid to promote CAT enzyme expression was compared with those of other CAT-containing plasmids.

Accepted May 2, 1989
Received March 29, 1989
2 To whom all correspondence should be addressed.
3 Present address: Laboratory of Molecular Embryology, Zoological Institute, Faculty of Science, University of Tokyo, Tokyo 113, Japan.
MATERIALS AND METHODS

Plasmid DNAs

Five different plasmids, pSV0CAT, pAd12.IXCAT, pAd12.E1aCAT, pA10CAT3m, and pSV2CAT were used throughout the experiments. pSV0CAT contains CAT gene and has a Hind III site in front of the CAT gene for experimental promoter insertion [6]. pAd12.IXCAT was constructed by inserting the promoter region (1.2 Kb) of polypeptide IX gene into Hind III site of pSV0CAT (Fig. 1) [5]. pAd12.E1aCAT was produced by inserting the promoter of E1a early gene of adenovirus type 12 into pSV0CAT (Fig. 1) [5]. pSV2CAT contains a CAT gene fused to a relatively strong promoter of SV40 early gene (Fig. 1) [6]. pA10CAT3m is a derivative of pSV0CAT, into which a polylinker was inserted [7].

Fig. 1. Maps of pAd12.IXCAT as compared with pAd12.E1aCAT and pSV2CAT. Thick lines indicate promoter inserted. IX, E, and 2 corresponds to pAd12.IXCAT, pAd12.E1aCAT, and pSV2CAT, respectively. Arrowheads outside circles denote Hind III sites, and those inside circles indicate approximate TATA box positions. Small arrows denote Hpa II sites. CAT denotes the site of CAT gene. These figures were drawn according to Krucek and Doerfler (5).

Plasmids were propagated, and DNAs were extracted as described previously by Tashiro et al. [8, 9]. Agarose gel electrophoresis showed that all the plasmid DNAs before injection consisted mainly of closed circular (c.c.) DNA, with little open circular (o.c.) DNA (Fig. 2).

Microinjection

Ovaries were digested for 3–4 hr with 500 μg/ml of collagenase in Barth’s solution (88 mM NaCl, 10 mM Hepes, pH 7.4, 1.0 mM KCl, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2) which contained 50 units/ml of penicillin and 50 μg/ml of streptomycin. Defolliculated oocytes at the stage 6 [10] were collected, and injected with ca. 20 nl of 100 μg/ml of DNA solutions. All oocytes injections were aimed at the germinal vesicle. Samples for CAT assay and DNA extraction were prepared from pools of 40 oocytes.

Fertilized eggs were obtained by artificial insemination [11], and after being dejellied in 2.5% thioglycollate (pH 8.0) [12], injected into the cytoplasm with ca. 20 nl of 100 μg/ml of DNA solutions in 1×MMR (0.1 M NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM Hepes, pH 7.4, 0.1 mM EDTA) that contained 5% Ficoll [8]. After injection, embryos were left in 1×MMR with 5% Ficoll, and transferred to 0.5×MMR when they reached the stage 6 [13]. Embryos were incubated until the blastula (stage 8.5), gastrual (stage 11) and neurula (stage 18–19) stages at 20–21°C. Ten embryos were used for a sample.

Unfertilized eggs were manually stripped out from the gravid female, dejellied, then incubated in modified Barths’ saline (88 mM NaCl, 1.0 mM KCl, 0.83 mM MgSO4, 0.34 mM Ca(NO3)2, 0.41 mM CaCl2, 7.5 mM Tris-HCl (pH 7.6), 2.4 mM NaHCO3). Eggs were injected into the cytoplasm with DNA as above [14], and left in the modified
Barths' saline at 20–21°C for either 12 or 24 hr. About 20 eggs were collected as a sample.

**CAT enzyme assay**

Oocytes, unfertilized eggs, and embryos were homogenized in 0.25 M Tris-HCl (pH 8.0), and supernatants equivalent to 10 eggs were mixed with 1 µCi of (14C)chloramphenicol (Amersham Corp.) and acetyl CoA. The mixture was incubated at 37°C for 2 hr, and was extracted twice with ethyl acetate. The extracts were spotted onto a thin layer plate with silica gel, and chromatographed for ca. 30 min in 95% chloroform-5% methanol. Gels were dried and autoradiographed usually for 3–5 days (for oocytes and embryos) or 1–2 months (for unfertilized eggs).

**RESULTS AND DISCUSSION**

Circular pAd12.IXCAT and other plasmids (pAd12.E1aCAT, pSV2CAT, pSV0CAT, pA10CAT3m) were injected into the oocyte nucleus at 2 ng/oocyte, and CAT enzyme activity was assayed after different periods of time. Southern blot analysis carried out with pSV2CAT as a probe showed that injected plasmids were stably preserved after 24 hr of incubation (data not shown).

CAT enzyme expression with pAd12.IXCAT was not greatly different from that with other four plasmids (pAd12.E1aCAT, pSV2CAT, pSV0CAT, and pA10CAT3m) at 7 (lanes 1 to 3), 20 (lanes 4 to 9) and 24 hr (lanes 10 to 12) (Fig. 3). Thus, as in the previous data which were obtained with chicken ovalbumin genes [16], all the circular genes were expressed equally actively.

Fertilized eggs were then injected with pAd12.IXCAT and four other circular plasmids at 2 ng/egg, and CAT enzyme activity was tested at different stages of development (Fig. 4). Under the conditions used, CAT enzyme activity was not detected at the blastula stage with plasmids that carried adenovirus promoters (lanes 10, 13), although pSV2CAT was expressed weakly as Etkin and Balcells [15] recently showed (lane 7). At the gastrula stage, however, all the plasmids were expressed at widely differing extents. Thus, pSV2CAT (lane 8) and pAd12.E1aCAT (lane 14) were strongly expressed, whereas pAd12.IXCAT was expressed only at a low level (lane 11), which was close to those of pSV0CAT (lane 2) and pA10CAT3m (lane 6). The results were essentially the same also at the neurula stage (Fig. 4).

DNAs were extracted from the above DNA-injected embryos at the blastula, gastrula and

---

**Fig. 3.** CAT enzyme assay in oocytes injected with circular plasmids. Oocytes were injected with 2 ng of circular plasmids and harvested at 7 hr (lanes 1 to 3), 20 hr (lanes 4 to 9) and 24 hr (lanes 10 to 12). Lanes 1, 8 and 10 (pSV0CAT); lanes 2, 4, 11 (pAd12.IXCAT); lanes 3, 5, 9, and 12 (pSV2CAT); lane 6 (pAd10CAT3m); and lane 7 (pAd12.E1aCAT). C, AC1, AC2, and AC3 are for chloramphenicol, 1-acetylated chloramphenicol, 3-acetylated chloramphenicol, and 1,3-diacetylated chloramphenicol, respectively.
neural stages, and Southern blot analysis was carried out using pSV2CAT as a probe. The results showed that injected pAd12.IXCAT as well as pSV0CAT and pAl0CAT3m was not degraded, but copy number of the injected DNAs increased by several fold. Therefore, the low activity of pAd12.IXCAT (Fig. 4) may not be due to the instability of this plasmid after injection.

Expression of CAT enzyme activity from circular pAd12.IXCAT and other plasmids was also tested after injection into unfertilized eggs at 2 ng/egg. As shown in Figure 5, very weak, but distinct CAT enzyme activity was observed at 12 hr only with pAd12.ElaCAT (lane 5) and pSV2CAT (lane 3), and the activity of other plasmids was either very faint (pAd12.IXCAT) (lane 2) or negligible (pSV0CAT, lane 1 and pA10CAT3m, lane 4). Thus, results obtained with unfertilized eggs were quite similar to those obtained with embryos, although the extent of the expression was quite low.

Circular plasmids were injected into fertilized eggs and RNAs were extracted from embryos at the gastrula stage to compare the level of CAT mRNA by Northern blot analysis [17] using CAT antisense RNA [18] as a probe. The results obtained showed that the level of the mRNA which migrated at 1.6 Kb CAT antisense RNA was roughly comparable to the CAT enzyme level (data omitted). Therefore, we assume that CAT enzyme activity may be roughly correlated to the activity of transcription of CAT genes in
embryonic cells.

As shown in Figure 1, the size of the promoter region of polypeptide IX gene used is almost twice as large as those of E1a protein and SV40 early genes. Nevertheless, the activity of CAT enzyme expression with pAd12.IXCAT that carried the promoter of polypeptide IX gene was as low as those of pSV0CAT and pA10CAT3m which do not contain the promoter.

pSV2CAT and pAd12.E1aCAT were known to contain a relatively strong enhancer element within their promoter region [5, 6]. However, the 5'-upstream region of the polypeptide IX gene of adenovirus has not been shown to contain an enhancer element [1, 2, 4], although the promoter of protein IX gene of adenovirus type 12 is known to contain TATA box and GC-rich region [4, 19]. The low activity of pAd12.IXCAT observed here in *Xenopus* embryos and unfertilized eggs suggests that the promoter of the protein IX gene of adenovirus type 12 may not contain an enhancer element.

**ACKNOWLEDGMENTS**

We thank Professor K. Yamana for his warm encouragement throughout the experiments. We also thank Dr. W. Doerfler for kind supply of CAT gene-containing plasmids. The present study was supported in part by a Grant-in-Aid for Scientific Research to K. S. (No. 61540523), a Grant-in-Aid for Cancer Research to K. H. (No. 62010095) from the Ministry of Education, Science and Culture of Japan, and grants from Takeda Science Foundation (1986) and The Naito Foundation (1987) to K. S.

**REFERENCES**


Virology, 90: 67–79.


17. Atschi, Y., Tashiro, K., Yamana, K. and Shiok-
