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Potential Activity of Transcriptional Promoter in the Replication Origin Region of Adenovirus type 5 DNA

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SUMMARY

The replication origin of adenovirus DNA has been located within the terminal 51 base-pair sequences at either terminus of a linear viral genome. We have examined transcriptional promoter activity of adenovirus replication origin. The test plasmids contain adenovirus type 5 left-terminal fragments placed upstream from the promoter-removed neomycin-resistant (neo) gene. Transcription and initiation sites of the neo gene were determined by S1 nuclease analysis of accumulated cytoplasmic RNA after short and long term transfections. The replication origin and origin-containing DNA fragments had promoter activity to direct transcriptional initiation in the outside direction of the viral genome. Major initiation sites were located 31 and 38 base-pair downstream from the first T of AT sequences of the minimal origin. Deletions in the origin region decreased the level of the neo gene transcript with a precise initiation site and increased levels of the neo gene transcripts with minor initiation sites found with a wild type plasmid. Thus, the origin region, probably AT sequences of the minimal origin, were required for positioning the initiation sites precisely. The results suggest that adenovirus replication origin contains proximal promoter sequences.

Key words : Adenovirus, Transcription, Replication origin

INTRODUCTION

An initiation of adenovirus (Ad) DNA replication takes place at either terminus of the linear viral genome (reviewed in reference 1) and requires the presence of special nucleotide sequence domains. At least three functionally distinct domains have been identified by mutation analyses of adenovirus type 2(Ad2) and 5(Ad5) origins(2, 3). Domain A (Ad5 nucleotides 1 to 18), referred to as the minimal origin, was absolutely required for origin function(4-8). The minimal origin contained a perfectly conserved AT-rich stretch ($^{5'}$GGTATATTAT$^{3'}$, nu-

cleotides 9 to 18) in human adenoviruses(9) that is similar to the TATA box sequence of promoters from higher eukaryote and virus genes(10). Domains B (nucleotides 19 to 40) and C (nucleotides 41 to 51) contained auxiliary sequences that efficiently increased the initiation activity of the minimal origin and are recognized by cellular proteins, the nuclear factor I (NF1)(11-15) and III (NFIII) (2, 3, 16), respectively. Addition of NF1 and NFIII factors efficiently increased an initiation reaction of Ad DNA replication *in vitro*(2, 14, 16). Recently, these replication factors of NF1 and NFIII have been identified as a CCAAT-binding transcription factor (CTF)(17) and as an ubiquitous octamer-motif binding transcription factor (OTF1/OCT1)(18), respectively. Thus, promoter elements including TATA box-like sequence and transcription factor recognition sequences resides in close proximity within Ad replication origin. We asked whether the replication origin and the origin-containing terminal fragments have promoter activity to direct transcriptional initiation and whether AT stretch of the minimal origin has a TATA box activity to position a site of transcription initiation approximately 30 base-pair (bp) downstream.

MATERIALS AND METHODS

Cell lines and culture

HeLa cells, Ad5 DNA-transformed human embryonic kidney cell line 293(19), rat embryonic fibroblast cell line 3Y1(20) and mouse embryonic teratocarcinoma stem cell line F9 were maintained in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum.

Plasmids

For construction of promoter-lacking neomycin-resistant gene (neo) plasmid (pUCOneo, Fig. 1), the neo transcription unit containing neo gene, SV40 splice signal and SV40 poly(A) addition signal was isolated from the pSV_2neo plasmid (21) by StuI and EcoRI digestions and inserted into the SmaI and EcoRI sites of pUC13 plasmid. For construction of pUCAneo and pUCABneo(Fig. 1), the Ad5 left-terminal 194 bp BamHI (nucleotide 1, with a BamHI linker)/RsaI (nucleotide 194) and 454 bp BamHI (nucleotide 1, with a BamHI linker)/PvuII (nucleotide 454) fragments were isolated from the pUC-498/45 plasmid(22). These fragments were converted into the BamHI fragments by linkers and cloned into the BamHI site of pUCOneo. The 194 bp Ad terminal fragment was cleaved out from pUCAneo by BamHI and SalI digestions and cleaved with MnlI or AccII to yied the MnlI (nucleotide 41)/SalI (nucleotide 194, with a SalI linker) or AccII (nucleotide 73)/SalI (nucleotide 194, with a SalI linker) fragments. These fragments were cloned between the BamHI and SalI sites of pUCOneo by

converting the MnlI and AccII sites into the BamHI site with a linker to yield pUCAd140neo and pUCAd173neo (Fig. 1). For construction of pUCmOrineo and pUCOrineo, Ad5 left-terminal 18 bp and 51 bp sequences with BamHI and SalI recognition sequences at the end were prepared by annealing the following chemically synthesized oligonucleotides: A (5' TCGACGGTATATTATTGATGATGCCG3') and B (5' GATCCGGCATCATCAATAATATACCG3'); C (5' TCGACCCT CATTATCATATTGGCTTCAATCCAAAATAAGGTATATTATTGATGATGC CG3') and D (5' GATCCGGCATCATCAATAATATACCTTATTTTGGATTGAA GCCAATATGATAATGAGGG3'). Double stranded oligonucleotides were phosphorylated and cloned between the BamHI and SalI sites of the polylinker of pUCOneo plasmid (Fig. 1). The Ad origin sequences in pUCmOrineo and pUCOrineo plasmids were confirmed by the method of Maxam and Gilbert(23). Plasmid pG2 contains a transcription unit of the rabbit β-globin gene(24) and was generously provided by P. Chambon.

DNA transfection and G418 selection

Transfection of HeLa, 293, F9 and 3Y1 cells were performed using the calcium-phosphate precipitation method(25). Briefly, cells in a 100 mm dish were split into five dishes 1 day before transfection and 20 μg of plasmid DNA (10 μg of test plasmid and 10 μg of pUC13 or pG2 reference plasmid) in 1 m*l* was added as calcium-phosphate co-precipitates into cells in a dish. After incubation for 6-7 hrs, cells were fed with fresh medium and the incubation was continued for an additional 42 hrs for short term transfection assay. HeLa cells were treated with 20% (v/v) glycerol in medium for 3 min. For long term transfection assay, cells in a dish were transfected with 3 μg of test plasmid and 17 μg of pUC13 plasmid, split into 10 dishes 2 days after transfection, and maintained for 3 weeks in selection medium containing following amounts of G418 (in an actual power): HeLa cells, 600 μg/m*l*; 293 cells, 600 μg/m*l*; F9 cells, 200 μg/m*l*; 3Y1 cells, 400 μg/m*l*.

RNA isolation and S1 analysis

Cytoplasmic RNA from cells was prepared by lysis in reticulocyte standard buffer (RSB: 10 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 10 mM NaCl) containing 0.5% NP-40 and SDS phenol-chloroform extraction of the supernatant after pelleting nuclei. When cells were transfected, the isolated cytoplasmic RNA was treated with 10 units of RQI DNase (Promega Biotec) in 300 μl of the supplyer's DNase digestion buffer at 37℃ for 20 min, followed by phenol-extraction and ethanol-precipitation. S1 analysis of neo gene transcripts was performed as described by the method of Weaver and Weissman(26). End-labeled DNA probe

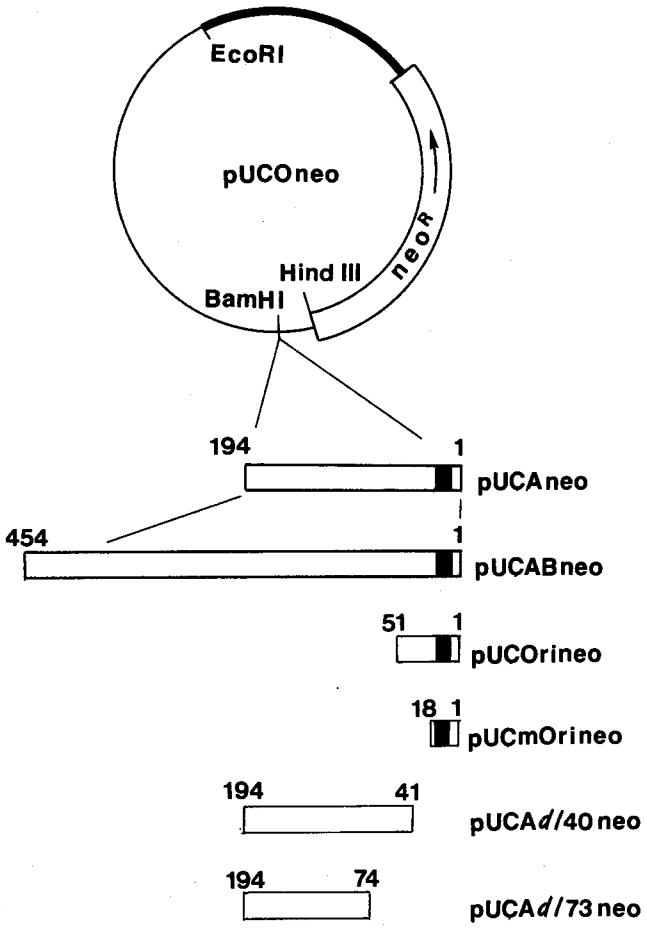

Fig. 1 Diagram of the test plasmids with Ad5 left-terminal fragments. The neo transcription unit containing bacterial neomycin-resistant gene (neo^R, open bar) and SV40 splice and poly(A) addition signals (black bar) was isolated from the plasmid pSV_2neo(21) by StuI and EcoRI and inserted into the SmaI and EcoRI sites of polylinker site of the pUC13 vector to yield pUCOneo. Plasmids pUCAneo and pUCABneo contain Ad5 left-terminal BamHI fragments of 194 bp (nucleotides 1 to 194) and 454 bp (nucleotides 1 to 454), respectively, inserted into the BamHI site of polylinker site of pUCOneo. Plasmids pUCmOrineo and pUCOrineo were obtained by cloning chemically synthesized doble stranded oligonucleotides containing Ad5 left-terminal 18 bp and 51 bp sequences into the BamHI and SalI sites of pUCOneo. Positions of the perfectly conserved AT-rich stretch in human Ad (nucleotides 9 to 18) are shown (closed bars). Plasmids pUCAd140neo and pUCAd173neo are mutants of the pUCAneo plasmid with deletions of the Ad5 left-terminal 40 and 73 bp sequences in the origin region.

($1-2\times10^5$ cpm) was incubated with 30-50 μg of cytoplasmic RNA in 30 μl of 80% formamide buffer(26) at 57℃ for 12-15 hrs, followed by digestion with 1,000 units per ml of S1 nuclease (Sigma Chemical Co.) at 30℃ for 1 hr. Hybridization was performed at 51℃ when short DNA was used as a probe. S1 protected products were precipitated with ethanol and resolved by electrophoresis in 5% or 8% polyacrylamide gel containing 8 M urea. For analysis of neo gene transcripts, 830 bp, 570 bp, and 376 bp BglII/PstI fragments, labeled at the BglII site, were isolated from pUCABneo, pUCAneo, and pUCOneo, respectively, and one of them was used as a probe depending upon the transfected test plasmid. To map 5' ends of the neo gene transcripts precisely, RNA was incubated with the following short DNA probes labeled at the FokI site of the neo gene: 281 bp FokI/NspI fragment of pUCAneo, 241 bp FokI/NspI fragment of pUCAd140neo, and 208 bp FokI/NspI fragment of pUCAd173neo. Rabbit β-globin-specific transcripts were quantitated with the SspI/BamHI fragment of pG2 plasmid, 5' end-labeled at the BamHI site(27). Probe DNA was prepared by labeling at 5' ends with T4 polynucleotide kinase and γ-^{32}P-ATP (3,000 Ci/mmole, NEN Dupont) and followed by digestion with either PstI or NspI.

RESULTS

We constructed recombinant plasmids as a test gene using bacterial neomycin-resistant gene (neo) which confers G418 (neomycin-analogue) resistant phenotype (neo$^+$) on mammalian cells(28). The pUCOneo (Fig. 1) is an SV40 enhancer and promoter-removed expression vector of the neo gene which was derived from the pSV_2neo plasmid(21). The pUCmOrineo and pUCOrineo contain the Ad5 minimal origin sequences (nucleotides 1 to 18) and origin sequences (nucleotides 1 to 51), respectively, inserted upstream of the neo gene of pUCOneo plasmid. The pUCAneo and pUCABneo contain Ad5 left-terminal 194 bp (nucleotides 1 to 194) and 454 bp (nucleotides 1 to 454) sequences, respectively. These constructs carry the Ad5 left terminus with 27 bp distance upstream from the 5' end (HindIII site) of the neo gene (Fig. 1). The pUCAd140neo and pUCAd173neo are mutants of the pUCAneo plasmid with deletions in the origin region.

Promoter activity of Ad5 left-terminal fragments:

(1) *Short term transfection assay*

To determine the promoter activity of Ad5 left-terminal 194 bp and 454 bp fragments, 293 cells were transfected with the pUCOneo, pUCAneo and pUCABneo plasmids. Transcription and initiation sites of the neo gene were determined by S1 analysis of accumulated cytoplasmic RNA after short term transfection.

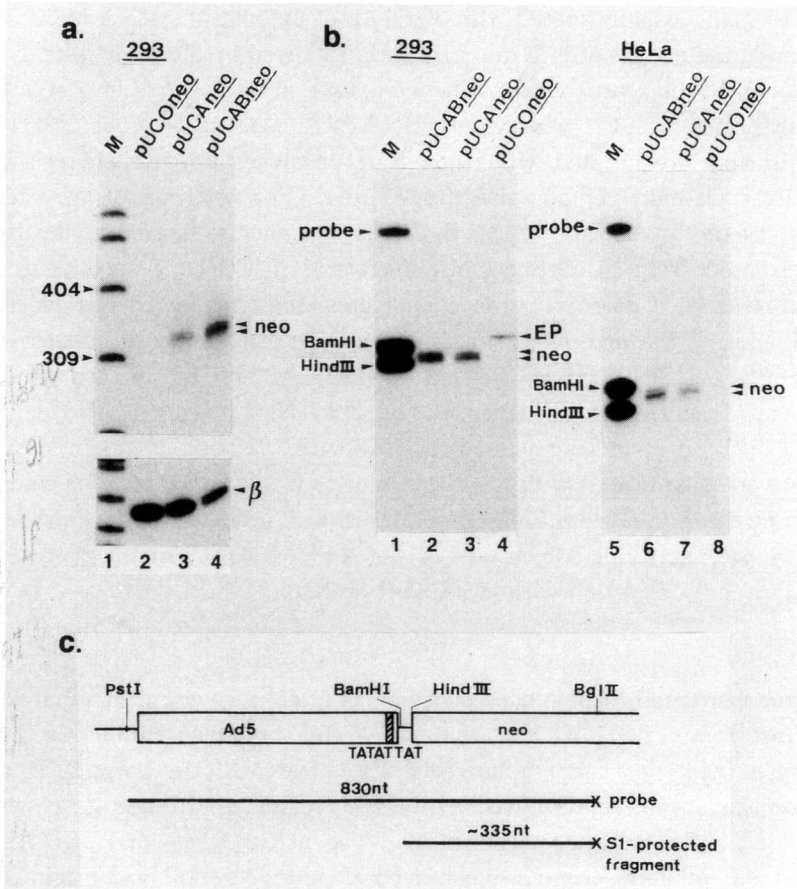

Fig. 2 S1 mapping of the 5' ends of neo gene transcripts in cells transfected with pUCAneo and pUCABneo plasmids.

a. Short term transfection assay: S1 analysis of neo gene transcripts from 293 cells transfected with pUCOneo (lane 2), pUCAneo (lane 3) and pUCABneo (lane 4); M (lane 1), marker HapII fragments of pBR322. For the estimation of transfection efficiency, pG2 plasmid containing a rabbit β-globin gene were cotransfected (β, lower panel). b. Long term transfection assay: S1 analysis of neo gene transcripts from 293 cells and HeLa cells transfected with pUCABneo (lanes 2 and 6), pUCAneo (lanes 3 and 7) and pUCOneo (lanes 4 and 8); M (lanes 1 and 5), 830 bp probe DNA and marker BamHI and HindIII fragments of 830 bp probe DNA. The band that corresponds to the end-point of the probe DNA (EP, lane 4) represents read through RNA initiating somewhere upsteam of the end-point of probe DNA. c. Probes: A 830 bp BglII/PstI fragments, 5' end-labeled at the BglII site, was isolated from pUCABneo and incubated with RNA from cells transfected with pUCABneo and pUCAneo. For pUCOneo transfection, a 376 bp BglII/PstI fragment of pUCOneo, 5' end-labeled at the BglII site, was used as a probe.

Neo gene-specific transcripts were detected in 293 cells transfected with plasmids containing the Ad DNA fragments (pUCAneo and pUCABneo), but not in 293 cells transfected with a plasmid containing no exogenous sequences (pUCOneo) (neo, Fig. 2a). The result is not due to the difference in amounts of the test plasmids incorporated into cells, because the transfection efficiency of pUCOneo was rather higher than those of pUCAneo and pUCABneo as revealed by levels of β-globin gene transcripts as an internal marker (β, Fig. 2a). Two closely migrated S1-protected fragments with sizes of approximately 335 nucleotides were generated, therefore positioning transcription initiation sites approximately 335 bp upstream from the labeled BglII site (Fig. 2c). Thus, initiation sites were located approximately 30 bp downstream from AT sequences of the minimal origin.

(2) *Long term transfection assay*

Promoter activity was tested in a form of plasmids integrated into cellular chromosomal DNA. HeLa cells and 293 cells were transfected with plasmids and maintained in selection media containing G418 for three weeks. Total of G418-resistant cell colonies (neo^+ cells) were collected, expanded, and subjected to S1 analysis (Fig. 2b). Two S1-protected fragments of approximately 335 nucleotides were again detected in either neo^+ cells derived from 293 or HeLa cells by transfection with pUCAneo and pUCABneo plasmids, but not with pUCOneo plasmid (Fig. 2b). S1-protected fragments migrated between the BamHI and HindIII markers, indicating the location of initiation sites between the BamHI and HindIII sites of test plasmids. The pUCOneo plasmid yielded a single S1-protected fragment with the same size as 376 bp probe DNA prepared from pUCOneo (Fig. 2b, lane 4). It may be due to reading through RNA initiating somewhere upstream of the end point (EP) of the probe DNA. The results indicated that Ad5 left-terminal 194 bp and 454 bp fragments had promoter activity to direct transcription initiation in the outside direction of the viral genome and that AT sequence of the minimal origin was a putative TATA box to position initiation sites approximately 30 bp downstream.

Transformed neo^+ cell lines were eatablished from individual G418-resistant cell colonies and neo gene-specific transcripts were analyzed by S1-nuclease method (Fig. 3). Neo^+ 293 cell lines, isolated by transfection with pUCAneo and pUCABneo, yielded two closely migrated S1-protected fragments of 335 nucleotides (Fig. 3a). Plasmid pUCOneo containing no exogenous sequences also induced neo^+ transformation. These neo^+ 293 cell lines yielded a S1-protected fragment with the same mobility as 376 bp probe DNA and/or a S1-protected fragment with higher mobility (Fig. 3a, lanes 2-4). The fast migrated fragment

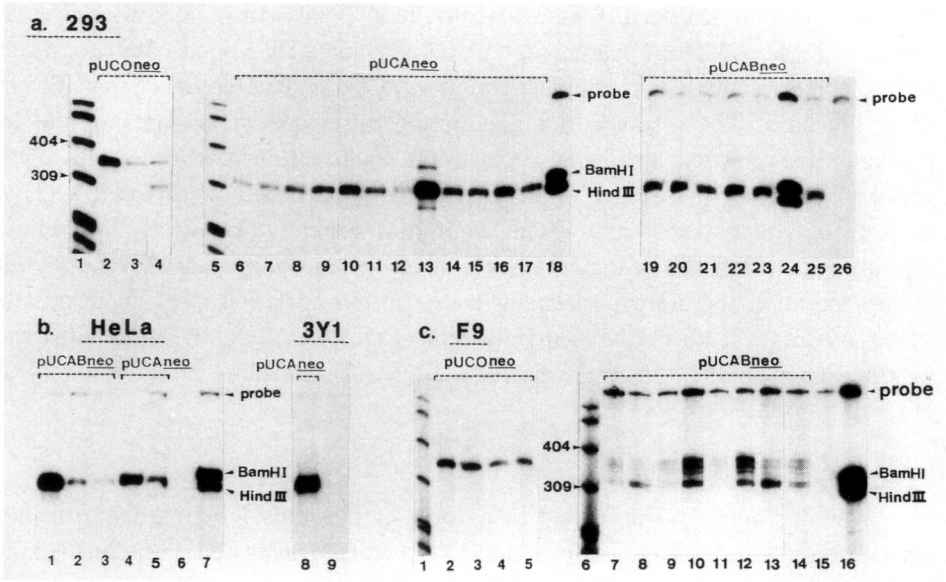

Fig. 3 S1 mapping of the 5' ends of neo gene transcripts in stably transformed neo$^+$ cell lines. a. 293 cell: RNA from neo$^+$ cell lines transformed with pUCOneo (lanes 2-4), pUCAneo (lanes 6-17) and pUCABneo (lanes 19-23 and lane 25); control RNA from 293 cells (lane 26); BamHI and HindIII marker fragments (lanes 18 and 24) of 830 bp probe DNA and HapII marker fragments of pBR322 (lanes 1 and 5). b. HeLa and 3Y1 cells: RNA from neo$^+$ cell lines transformed with pUCABneo (lanes 1-3) and pUCAneo (lanes 4, 5 and 8); control RNA from HeLa cells (lane 6) and 3Y1 cells (lane 9); BamHI and HindIII marker fragments of 830 bp probe DNA (lane 7). c. F9 cell: RNA from neo$^+$ cell lines transformed with pUCABneo (lanes 7-14) and pUCOneo (lanes 2-5); control RNA from F9 cells (lane 15); BamHI and HindIII marker fragments of 830 bp probe DNA (lane 16) and HapII fragments of pBR322 (lanes 1 and 6). RNA was incubated with the 830 bp or 376 bp BglII/PstI fragments, as shown in Fig. 2C.

may represent neo gene transcripts initiating at cellular sequences flanked with the integrated plasmid DNA. Neo$^+$ HeLa cell lines, isolated by transfections with pUCABneo and pUCAneo, produced a S1-protected fragment of approximately 335 nucleotides (Fig. 3b). A neo$^+$ 3Y1 cell line, isolated by transfection with pUCAneo plasmid, also produced a S1-protected fragment of approximately 335 nucleotides (Fig. 3b). Neo$^+$ F9 cell lines transformed with pUCABneo plasmid yielded at least five S1-protected fragments: approximately 335 nucleotide fragment migrated between the BamHI and HindIII markers and 355, 370, and 385 nucleotides (Fig. 3C). Neo$^+$ F9 cell lines transformed with pUCOneo generated a S1-protected fragments with the same size as 376 bp probe DNA (Fig. 3C). The results indicated that the Ad5 left-terminal fragments were an active

promoter in different cells.

Promoter activity of Ad5 replication origin:

(1) *Short term transfection assay*

We have examined whether the minimal origin itself and the origin itself have an ability to direct transcription initiation. Cells(293) were transfected with pUCmOrineo and pUCOrineo plasmids which contain 18 bp sequences of the minimal origin and 51 bp sequences of the origin, respectively (Fig. 1). Cytoplasmic RNA was isolated 48 hr later and neo gene-specific transcripts were determined by S1 analysis using short probes labeled at the FokI site. S1-resistant products were resolved in sequencing gels to map initiation sites precisely (Fig. 4). The pUCAneo plasmid yielded two prominent S1-protected fragments with sizes of 123 and 116 nucleotides that migrated between the BamHI and HindIII markers and at least seven minor S1-proteceted fragments (short and long exposure autoradiographes in Fig. 4, lane 4). Therefore, major initiation sites were mapped at AGG and TG sequences between the cleavage sites of BamHI and HindIII (Fig. 5), which were in good agreement with the previously determined initiation sites (Fig. 2, 3). These sites were located 31 and 38 bp downstream from the first T residue of AT sequences of the minimal origin and were referred to as the positions +1 and +8, respectively. Minor initiation sites were mapped at the following positions relative to the major site: +14, −11, −21 (Ad5 nucleotide 7), −31 (nucleotide 17), −49 (nucleotide 35) and around −68 (nucleotide 54) and −98 (nucleotide 84). The pUCOrineo plasmid with the terminal 51 bp sequences yielded very low, but detectable levels of S1-protected fragments of 116 and 123 nucleotides (arrowheads in longer exposure autoradiograph in Fig. 4, lane 2). The pUCmOrineo plasmid with the terminal 18 bp sequences did not yield S1-protected fragments of 116 and 123 nucleotides (longer exposure autoradiograph in Fig. 4, lane 3). The pUCOneo plasmid yielded S1-protected fragments migrated around the BamHI cleavage site (Fig. 4, lane 1). This represents neo gene transcripts initiating at vector DNA sequences upstream of the BamHI site because these RNA species could hybridize with the probe DNA within the limited region from the labeled FokI site until BamHI site. Similarly, most slowly migrating fragments generated with pUCOrineo and pUCmOrineo represents neo gene transcripts initiated at vector DNA sequences (Fig. 4, lanes 2 and 3). The results indicated that the replication origin supported transcription initiation at precise sites with very low efficiency while the minimal origin failed to support initiation. The results also indicated that upstream sequences of the origin were required for active transcription. Next, 293 cells were transfected with pUCAd140neo and pUCAd173neo, mutants of the pUCAneo plasmid with

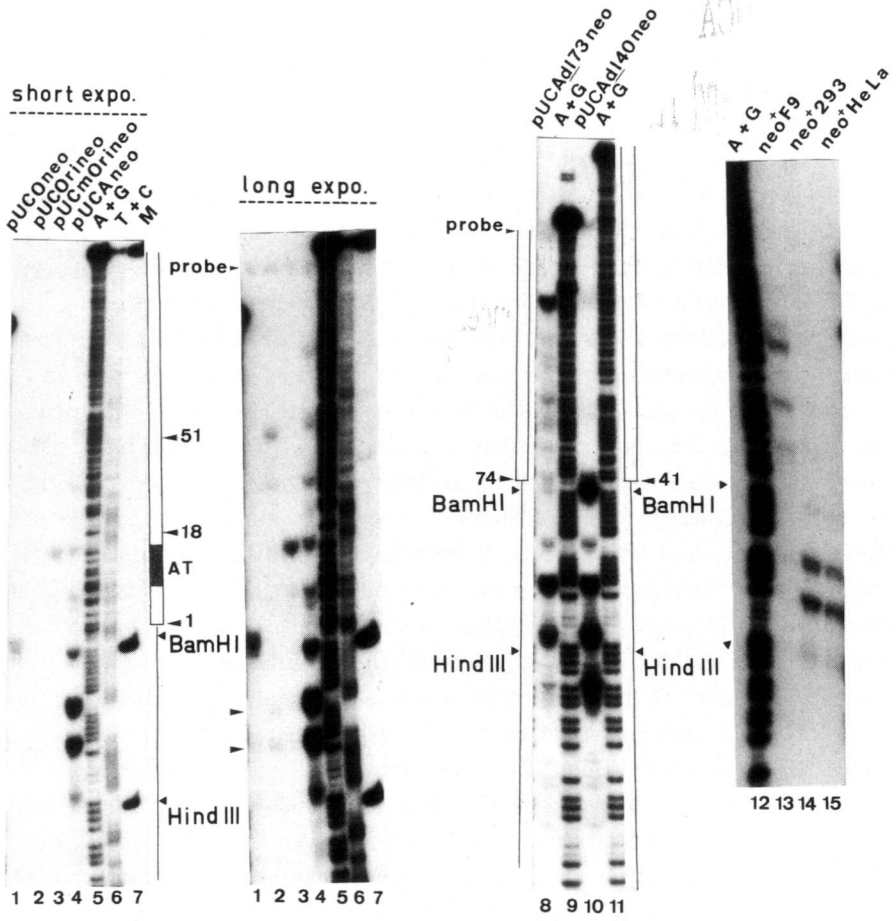

Fig. 4 Detailed S1 mapping of the 5' ends of neo gene transcripts in 293 cells transfected with the pUCAneo, pUCmOrineo, pUCOrineo, pUCAd140neo, and pUCAd173neo plasmids. RNA from 293 cells transfected with pUCAneo (lane 4), pUCmOrineo (lane 3), pUCOrineo (lane 2), pUCOneo (lane 1), pUCAd140neo (lane 10), and pUCAd173neo (lane 8); RNA form neo$^+$ 293 cells (lane 14) and neo$^+$ HeLa cells (lane 15) transformed with pUCAneo plasmid; RNA from a neo$^+$ F9 cell line #13 transformed with pUCAneo (lane 13). A+G and T+C are A+G and T+C sequencing lanes(2) of the probe DNAs depending upon the transfected plasmids. M (lane 1), marker BamHI and HindIII fragments of the probe DNA prepared from pUCAneo plasmid. RNA was incubated at 51 C with the the following probes labeled at the FokI site: 281 bp FokI/NspI fragment of pUCAneo, lanes 1, 2, 3, 4, 13, 14, and 15; 241 bp FokI/NspI fragment of pUCAd140neo, lane 10; 208 bp FokI/NspI fragment of pUCAd173neo, lane 8. Open bars alongside the autoradiographes indicate positions of Ad5 nucleotide sequences. AT (shaded) is AT sequences of the minimal origin which is perfectly conserved in human adenoviruses(9). Long exposure: fivefold-longer exposure of lanes 1 to 7.

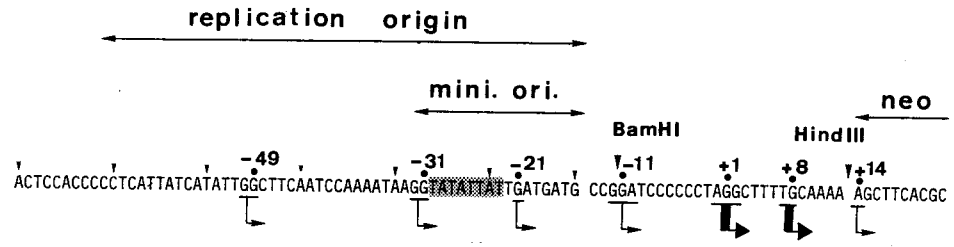

Fig. 5 Location of initiation sites of the neo gene transcripts on the test plasmid. The sequences of the test plasmid around the boundary of Ad5 left-terminal 60 bp sequences and 5' end of the neo gene are shown. Sequences of 27 nucleotides at the boundary are derived from the plasmids of pUC13 and pSV_2neo. AT strech of the minimal origin, perfectly conserved in human adenoviruses(9), was shaded. Major and minor initiation sites were shown by thick and thin arrows, respectively, with numbers which indicate relative positions to the major site at position +1.

deletions of the left-terminal 40 and 73 bp sequences, respectively. Either plasmid yielded several S1-proteceted fragments with varying band intensities, indicating multiple initiation sites (Fig. 4, lanes 8 and 10). Deletion mutant of the terminal 40 bp sequences yielded high levels of neo gene transcripts initiating at positions −11, +8, +14, and +21 (Fig. 4, lane 10). Initiations at positions +8 and +14 and approximately −135 (Ad5 nucleotide 121) were clearly detected with a deletion mutant lacking the leftterminal 73 bp sequences (Fig. 4, lane 8). Some initiation sites were common to the mutants and a wild type pUCAneo plasmid but with varying intensities (Fig. 4, compare lanes 4, 8 and 10). However it should be noted that one of the major initiation sites with a wild type plasmid (position +1) was inactivated to a large extent with both deletion mutants. Furthermore, deletion plasmids supported significant initiations of transcription at a new site (position −135) and at minor sites found with a wild type plasmid (positions −11, +14, and +21, Fig. 4). The results indicated that the terminal 40 bp sequences contain TATA box-like element to direct initiation at precise sites. We believe that AT sequences (TATATTAT, Ad5 nucleotides 9 to 16, Fig. 5) of the minimal origin has TATA box activity. Neo^+ 293 and HeLa cells, transformed with pUCAneo or pUCABneo, produced neo gene transcripts with 5' ends located at positions +1 and+8 (Fig. 4, lanes 14 and 15). A neo^+ F9 cell line produced transcripts initiating further upstream (Fig. 4, lane 13). These sites were positions −21, −31, and −49 located inside of the viral genome and were common to minor initiation sites identified by short term transfection assay (Fig. 4, lanes 4 and 13).

(2) *long term transfection assay*

Promoter activity of the rplication origin sequences was confirmed by long term transfection assay. HeLa cells were transfected with pUCmOrineo and pUCOrineo plasmids and G418-restant cell colonies were screened in selection media. First, we could not obtain any G418-resitant cell colonies. Therefore, we carried out G418 selection under less stringent conditions; two-thirds (400 $\mu g/ml$) of normal amount of G418 and shortened culture in selection media (about 10 days). This enabled us to isolate several colonies and determine initiation sites of neo gene transcripts by S1 analysis. Most of eight neo$^+$ cell lines, isolated by transfection with pUCOrineo, clearly yielded two or three S1-protected fragments migrated between the BamHI and HindIII markers (Fig. 6, lanes 6, 7, 9, 10, 11, 12 and 13), while these fragments were not detected in two neo$^+$ cell

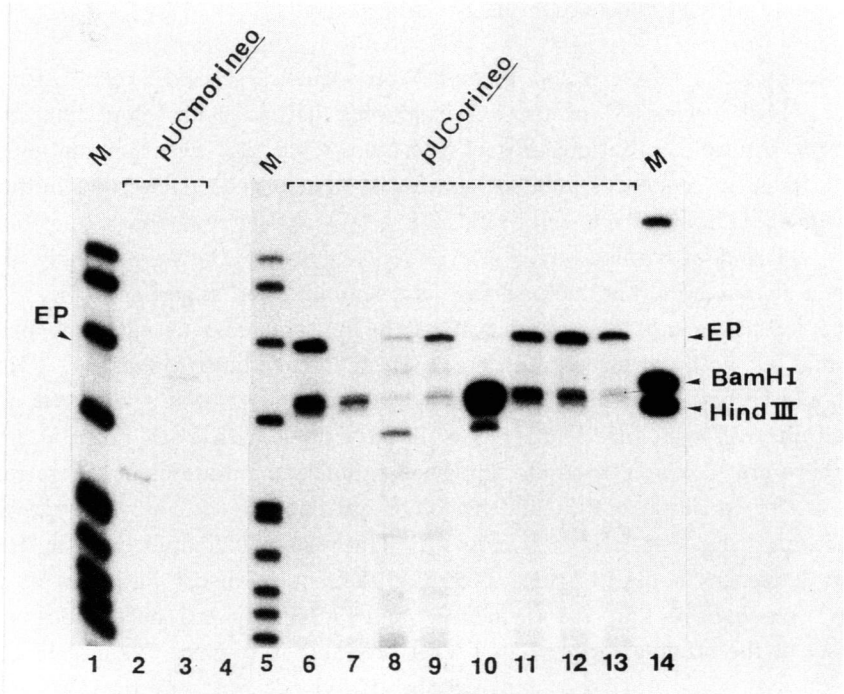

Fig. 6 S1 mapping of the 5' ends of neo gene transcripts in neo$^+$ HeLa cell lines transformed with the pUCOrineo and pUCmOrineo plasmids. RNA from neo$^+$ HeLa cell lines transformed with pUCmOrineo (lanes 2 and 3) and pUCOrineo (lanes 6-13); control RNA from HeLa cells (lane 4); BamHI and HindIII marker fragments of 830 bp probe DNA (lane 14) and HapII fragments of pBR322 (lanes 1 and 5). RNA was incubated with the following probes labeled at the BglII site: 374 bp BglII/PstI fragment of pUCmOrineo, lanes 2-4; 407 bp BglII/PstI fragment of pUCOrineo, lanes 6-13. EP, end point of probe DNA.

lines isolated by transfection with pUCmOrineo (Fig. 6, lanes 2 and 3). The difference in levels of the neo gene transcripts might be due to sites where the test neo plasmids were integrated into cellular DNA. Most of the cell lines generated S1-protected fragment with the same mobility as that of the probe DNA (EP in Fig. 6, lanes 3, 6, 8, 9, 11, 12 and 13). They may represent read through RNA initiating somewhere upstream of the end point (EP) of the probe DNA. Thus, long term transfection assay clearly indicated that the terminal 51 bp sequences, replication origin itself, have an ability to direct transcriptional initiation precisely.

Increased neo$^+$ transformation by insertion of the Ad left-terminal fragments

As a second test of promoter activity of the Ad left-terminal fragments, cells were transfected with test plasmids of pUCOneo, pUCAneo and pUCABneo (Fig. 1) and transformed neo$^+$ cells were selected in G418-containing media. Promoter activity was largely estimated by counting neo$^+$ cell colonies. Insertion of the 194 bp and 454 bp left-terminal fragments into the pUCOneo plasmid increased the efficiency of neo$^+$ transformation in all cell lines tested, as judged by comparison of pUCAneo, pUCABneo and pUCOneo plasmids (Table 1). In 293 and F9 cells, the efficiency was much higher than those in HeLa and 3Y1 cells. It might be due to an activation of promoter and/or stabilization of transfected DNA in 293 and F9 cells. Neither plasmid carrying the minimal origin nor origin sequences alone produced distinct G418-resistant neo$^+$ cell colonies under standard selection conditions, although positive control pUCAneo and pSV$_2$neo plasmids yielded many neo$^+$ cell colonies (data not shown). It may be due to a low level of neo gene expression because promoter activity of the origin sequences is much weaker than those of the left-terminal 194 bp sequences (Fig. 4).

Table 1 *Neo$^+$ transformation activity of pUCOneo, pUCAneo, and pUCABneo in 293, F9, HeLa and 3Y1 cells.*

transfected plasmids	number of neo$^+$ colonies/μg DNA			
	293	F9	HeLa	3Y1
pUCOneo	6	17	0.3	0
pUCAneo	170	197	2	0.17
pUCABneo	132	ND	0.5	1.7
pSV$_2$neo	739	119	37	134

Cells were transfected with plasmids and subjected to G418 selection as described in Methods. After 3 weeks, G418-resistant cell colonies (neo$^+$) were stained with Giemsa's solution and counted. Numbers are averages in 2 to 4 experiments. ND: not determined.

DISCUSSION

We confirmed the previous findings(29, 30) that the Ad5 left and right-terminal fragments had promoter activity to direct transcriptional initiations in the outside direction of the viral genome. In this study, promoter activity was observed in either episomal or integrated form of transfected plasmids and in cells from different species. Furthermore, we have examined transcriptional significance of replication origin sequences. The results indicated that replication origin itself (the terminal 51 bp sequences) had very low, but detectable levels of promoter activity while it was not detected with the minimal origin (the terminal 18 bp sequences). Deletion analysis of origin sequences indicated that origin sequences were required for positioning initiation sites precisely. Major initiation sites were located 31 and 38 bp downstream from AT sequences of the minimal origin, in good agreement with those of *in vitro* transcripts(29, 30). However short term transfection assay in HeLa cells revealed different initiation sites, located at Ad5 nucleotides 2 and 39 on the viral genome(30). In our experiments initiations inside of the viral genome were clearly detected in F9 cells but not in 293 and HeLa cells. Comparison of promoter activity in the left-terminal 18, 51, and 194 bp fragments suggest that the replication origin region contains proximal promoter sequences where an initiation complex would be formed by interaction with basic transcription factors such as TFIID and RNA polymerase II. Upstream region of the replication origin may contain transcription activating elements. Recently, we and others have identified in this region several binding sites of HeLa cell nuclear proteins: SP1 (Ad5 nucleotides 79 to 108), ATF (nucleotides 49 to 71, 85 to 108, and 161 to 180) and E1A enhancer factor (E1A-F or EF-1A, nucleotides 105 to 124, and 153 to 165)(22, 31). Although these sequences appear to be dispensable for initiation of Ad DNA replication (3-5, 7, 12, 15), they might be activators in the origin-directed transcription. In the initiation reaction of Ad DNA replication, the minimal origin is the site for the 80 kD terminal protein precursor -dCMP/DNA polymerase complex, both of which are products of viral gene (for reviews, see ref. 1, 32). Ad minimal origin also interacted with a HeLa cell nuclear protein (origin recognition protein A) and possibly an additional protein, although functions of these cellular proteins have not yet been characterized(2). As judged by in vitro competition binding assay, a common or related factor may interact with Ad minimal origin and proximal E1A promoter containing CCAAT and TATA boxes of the Ad5 E1A gene(22). Our study raised the possibility that minimal origin-binding factor(s) might be TATA box factor(s). The minimal (or core) origin of simian virus 40(SV40) also contains an AT element of 17 bp which overlapped TATA box of the viral

early gene(33, 34). It is unlikely in a competition fashion that a HeLa cell protein, identified by binding to the SV40 AT element, is related to TATA box factor(s) (ref. 35, our unpublished data). Biochemical characterization of these AT stretch-binding proteins will be required to determine a relationship between the Ad minimal origin and TATA box factors.

It is likely that initiation of Ad DNA replication does not require transcription around the replication origin, because it is not affected by α-amanitin, an inhibitor of RNA polymerases(32). Transcriptional promoter activity found in this study was in the outside direction of the viral genome. There is no more Ad DNA left on which to transcribe the RNA. Alternatively, it is possible that cellular transcription factor may facilitate bindings of Ad replication proteins to the replication origin. Ad replication origin contains the binding sites for NF1/CTF and NFIII/OTF-1 factors which have an ability to activate both replication of Ad DNA and transcription of some cellular genes(17, 18). Thus, it is assumed that transcriptional promoter elements containing the TATA box, NF1/CTF site, and NFIII/OTF-1 site were subverted by adenovirus for the replication of its own DNA. The *in vitro* reconstitution experiments with purified proteins will be required to understand the dual role of these transcription factors as initiators of transcription and replication.

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REFERENCES

1. KELLY, T. J., Jr.: In: GINSBERG H. S., The adenoviruses, 271-305, Plenum Publishing Corp., New York. (1984)
2. ROSENFELD, P. J., O'NEILL, E. A., WIDES, R. J. and KELLY, T. J.: **Mol. Cell. Biol. 7**, 875-886 (1987).
3. WIDES, R. J., CHALLBERG, M. D., RAWLINS, P. R. and KELLY, T. J., Jr.: **Mol. Cell. Biol. 7**, 864-874 (1987).
4. CHALLBERG, M. D. and RAWLINS, D. R.: **Proc. Natl. Acad. Sci. USA 81**, 100-104 (1984).
5. HAY, R. T.: **EMBO J. 4**, 421-426 (1985).
6. LALLY, C., DORPER, T., GROGER, W., ANTOINE, G. and WINNACKER, E.-L.: **EMBO J. 3**, 333-337 (1984).
7. TAMANOI, F. and STILLMAN, B. M.: **Proc. Natl. Acad. Sci. USA 80**, 6446-6450 (1983).
8. van BERGEN, B. G. M., van der LEY, P. A., van DRIEL, W., van MANSFELD, A. D. M. and van der VLIET, P. C.: **Nucleic Acids Res. 11**, 1975-1989 (1983).

9. SHINAGAWA, M., IIDA, Y., MATSUDA, A., TSUKIYAMA, T. and SATO, G.: **Gene 55**, 85-93 (1987).
10. BREATHNACH, R. and CHAMBON, P.: **Annu. Rev. Biochem. 50**, 349-383 (1981).
11. de VRIES, E., van DRIEL, W., TROMP, M., van BOOM, J. and van der VLIET, P.C.: **Nucleic Acids Res. 13**, 4935-4952 (1985).
12. GUGGENHEIMER, R.A., STILLMAN, B.W., NAGATA, K., TAMANOI, F. and HURWITZ, J.: **Proc. Natl. Acad. Sci. USA 81**, 3069-3073 (1984).
13. LEEGWATER, P.A.J., van DRIEL, W. and van der VLIET, P.C.: **EMBO J. 4**, 1515-1521 (1985).
14. NAGATA, K., GUGGENHEIMER, R.A. and HURWITZ, J.: **Proc. Natl. Acad. Sci. USA 80**, 6177-6181 (1983).
15. RAWLINS, D.R., ROSENFELD, P.J., WIDES, R.J., CHALLBERG, M.D. and KELLY, T.J., Jr.: **Cell 37**, 309-319 (1984).
16. PRUIJIN, G.J.M., van DRIEL, W. and van der VLIET, P.C.: **Nature 322**, 656-659 (1986).
17. JONES, K.A., KADONAGA, J.T., ROSENFELD, P.J., KELLY, T.J., Jr. and TJIAN, R.: **Cell 48**, 79-89- (1987).
18. O'NEILL, E., FLETCHER, C., BURROW, C.R., HEINTZ, N., ROEDER, R.G. and KELLY, T.J.: **Science 241**, 1210-1213 (1988).
19. GRAHAM, F.L., SMILEY, J., RUSSELL, W.C. and NAIRN, R.: **J. Gen. Virol. 39**, 59-77 (1977).
20. KIMURA, G., ITAGAKI, A. and SUMMERS, J.: **Int. J. Cancer 15**, 694-706 (1975).
21. SOUTHERN, P.J. and BERG, P.: **J. Mol. Appl. Gen. 1**, 327-341 (1982).
22. YOSHIDA, K., NARITA, M. and FUJINAGA, K.: **Nucleic Acids Res. 17**, 10015-10034 (1989).
23. MAXAM, A.M. and GILBERT, W.: **Methods Enzymol. 65**, 499-560 (1980).
24. HEN, R., BORRELLI, E., FROMENTAL, C., SASSONE-CORSI, P. and CHAMBON, P.: **Nature 321**, 249-251 (1986).
25. GRAHAM, F.L. and van der EB, A.J.: **Virology 52**, 456-467 (1973).
26. WEAVER, R.F. and WEISSMAN, S.: **Nucleic Acids Res. 7**, 1175-1193 (1979).
27. YOSHIDA, K., VENKATESH, L.K., MOHAN, K. and CHINNADURAI, G.: **Genes Dev. 1**, 645-658 (1987).
28. COLBERE-GARAPIN, F., HORODNICEANU, F., KOURILSKY, P. and GARAPIN, A.C.: **J. Mol. Biol. 150**, 1-14 (1981).
29. MATSUMOTO, K., NAGATA, K., YAMANAKA, K., HANAOKA, F. and UI, M.: **Biochem. Biophys. Res. Commun. 164**, 1212-1219 (1989).
30. OOYAMA, S., IMAI, T., HANAKA, S. and HANDA, H.: **EMBO J. 8**, 863-868 (1989).
31. BRUDER, J.T. and HEARING, P.: **Mol. Cell. Biol. 9**, 5143-5153 (1989).
32. DEPAMPHILIS, M.L.: **Cell 52**, 635-638 (1988).
33. BENOIST, C. and CHAMBON, P.: **Nature 290**, 304-310 (1981).
34. GHOSH, P.K., LEBOWITZ, P., FRISQUE, R.J. and GLUZMAN, Y.: **Proc. Natl. Acad. Sci. USA 78**, 100-104 (1981).
35. BAUR, C.-P. and KNIPPERS, R.: **J. Mol. Biol. 203**, 1009-1019 (1988).