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Analysis of the rat JE gene promoter identifies an AP-1 binding site essential for basal expression but not for TPA induction

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ABSTRACT

We have cloned the immediate-early serum-reponsive JE gene from the rat in order to study the regulation of this gene. We show that sequences of the JE promoter region confer serum-inducibility to a reporter gene. Analysis of the promoter in transient assays reveals that: i) the −141/−88 region is required for the response to the phorbol ester TPA, ii) the −70/−38 region is essential for basal activity. This latter region harbors the sequence TGACTCC, which resembles the consensus site for AP-1 binding, TGACTCA. DNA-protein binding assays indicate that the JE AP-1 site and the consensus AP-1 site have an overlapping but not identical binding spectrum for AP-1 proteins. Our data suggest that the inability of some AP-1 sites to respond to TPA is caused by subtle differences in affinity for AP-1 proteins.

INTRODUCTION

Cellular proliferation is a complex process involving temporal expression of cellular genes (reviewed in: 1). A number of genes, which are activated instantly after mitogen treatment, have been identified and cloned (e.g. 2, 3, 4). These so-called immediate-early (IE) genes share several characteristics. First, the genes are activated transcriptionally (3, 4, 5, 6). Secondly, the induction does not require new protein synthesis (2, 3, 7). And thirdly, the corresponding mRNAs have short half-lives (4, 8, 9). Most interestingly, some of the IE genes, e.g. the c-*fos*, the c-*myc* and the c-*jun* genes, are proto-oncogenes (for reviews: 10, 11, 12).

One of the cellular IE genes is the JE gene, which was first cloned as a platelet-derived-growth-factor(PDGF)-inducible cDNA from murine Balb/c 3T3 cells (2). Induction by PDGF is at the transcriptional level (6). A variety of other agents, like the phorbol ester TPA, epidermal growth factor, interferon-gamma, interleukin-1 and poly(I-C) have also been shown to induce JE mRNA expression in certain cell types (13, 14, 15, 16). The induction of JE has the same kinetics as c-*myc* induction. JE mRNA has a short half-life of approximately 70 min (6, 17). Its 25-kDa protein product is glycosylated and probably secreted. The precise function of the JE product is not known, but a cytokine-like role has been suggested (15).

We have previously shown that JE mRNA levels are severely reduced in cells transformed by the adenovirus (Ad) E1A region but not in cells transformed by other oncogenes (18). Similar to activation by mitogens, repression by E1A is at the transcriptional level (17). Both phenomena probably result from regulation of JE promoter activity. To understand the mechanism of regulation it is necessary to have a detailed knowledge of the JE gene. In this paper we report the molecular cloning and characterization of the rat JE gene. The JE promoter can render a reporter gene responsive to serum. Using transient expression assays we show that a stretch of only 70 bp upstream of the JE cap site are sufficient for the basal promoter activity. An essential element within this minimal promoter has strong resemblance to the consensus sequence for AP-1 binding. However, transient assays show that TPA induction requires an element upstream of the minimal promoter and *in vitro* binding assays indicate that the putative JE AP-1 site and the consensus AP-1 site bind different cellular factors.

MATERIALS AND METHODS

Materials

Sera and tissue culture media were obtained from GIBCO (Grand Island, N.Y.). All tissue culture plastics were purchased from Greiner (FRG). Radionucleotides and [^{14}C]-chloramphenicol were from Amersham International (U.K). Restriction enzymes were purchased from Pharmacia Biotechnology LKB (Uppsala, Sweden).

Cell culture and transfections

HeLa TK$^-$, Rat-1 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 300 μg/ml L-glutamine, 10% fetal calf serum and antibiotics. Serum deprivation and subsequent stimulation of cultures has been described before (17). Clonal NIH 3T3-transformants were obtained by cotransfecting pBA-JE-1200 or pBA-RSV with pSV2-neo as described (18). Transient transfection of HeLa

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TK$^-$ and Rat-1 cells was performed as described (19) using 10 μg of the CAT plasmids per transfection. One μg pRSV-hGH was included to standardize for transfection efficiencies. Human growth hormone levels were determined with a RIA kit (Diagnostics Products, Los Angeles, CA). Efficiencies never varied more than two-fold. CAT activity was assayed as described (20).

DNA cloning and sequence analysis

Chromosomal DNA from Ad12-transformed baby rat kidney cells was isolated by standard procedures. 330 μg of DNA was digested with subsequently *EcoR*I, *Sca*I and *Stu*I, fractionated on a 0.6% low-melting agarose gel and the gel slice containing the JE fragment was identified by blotting analysis as described (18). Part of this fraction was ligated to *EcoR*I-digested lambda-EMBL4 arms, packaged and plated on *E. coli* NM539 bacteria as described (21). Further manipulations were performed as in (22). Screening of 6×10^4independent plaques with the murine JE cDNA probe yielded four positives. Restriction analysis of these clones with *EcoR*I, *Xba*I, *Hin*dIII, *Sst*I and *Pst*I indicated that they have identical inserts. The 11-kb *EcoR*I-insert of clone gJE 1-1 was subcloned in pIC20H (23) to give pgJE1. The terminal 4.5-kb *EcoR*I-*Sph*I fragment, carrying the entire JE gene, was subcloned in M13 mp18 and mp19 for sequence analysis. Smaller portions of this fragment were also subcloned in M13 vectors. A panel of *Bal*31-deletion clones was prepared either from the *Bgl*II site at +54 towards the 5'-end (B-clones) or to the 3'-end (C-clones) or from the *BglII* site at +1318 to the 5'-end (A-clones). The DNA sequence was determined by the dideoxy chain termination method (24) using either M13- or sequence-specific primers.

All JE promoter-CAT constructs are based on pBA-CAT (25). The plasmids JE-2600, JE-1200, JE-543, JE-328 and JE-173 were obtained by inserting different restriction fragments, all ending at +59, into the *Hin*dIII site by use of a 12-bp linker. AP-1-site mutations were introduced in a M13 clone by the gapped-duplex DNA method (26) using specific oligonucleotides. Mutated fragments were sequenced completely and recloned into JE-543. Clones JE-141, JE-88, JE-70, JE-38, JE-24 and JE+4 were obtained by *Bal*31 digestion of clone JE-1200 from the *Sst*II site, and subsequent ligation of an *Xba*I linker. This resulted in the deletion from −1000 till the indicated position, identified by sequencing the CAT construct. Plasmid pBA-RSV contains the 581 bp *Nde*I-*Hin*dIII RSV-LTR fragment from pRSV-neo (27). RSV-5E1A was constructed by inserting the RSV-LTR fragment upstream of Ad5 E1A-region (position 509 to 1833). E1A promoter sequences were deleted by *Bal*31 digestion to position 509 (A.G. Jochemsen and R. Offringa, unpublished results). The CAT plasmids carrying the collagenase (−517/+63) or the TRE-col/TK promoter were kind gifts of Dr. P. Herrlich (Karlsruhe, FRG) and are described (19).

RNA analyses

RNA was isolated as described (18). Nuclease S1-mapping experiments with radiolabelled single-stranded probes were carried out essentially according to (28). Primer extension was performed as described (29) using the oligonucleotide 5'-CAGGCCCAGAAGCGTGAC-3', complementary to positions +105 to +88, as a 5'-labelled probe. RNase protection using a JE-CAT RNA probe was performed as described (30).

Computer analyses

Sequences were analysed and compared using the Wisconsin Software package (31). The murine JE sequence (accession number M19681, M19862) was obtained from the GenBank repository. The nucleotide sequence data of the rat JE gene will appear in the EMBL Data Library under accession number X17053.

DNA-protein binding assay

Cellular extracts were prepared essentially by repeatedly freeze-thawing, as will be described elsewhere. Oligonucleotides were annealed and 5'-labelled to an activity of 3.3×10^7 cpm/pmol (JE AP-1) or 3.4×10^7 cpm/pmol (collagenase AP-1). Formation of protein-DNA complexes and separation in 4% polyacrylamide gels were performed as described (32).

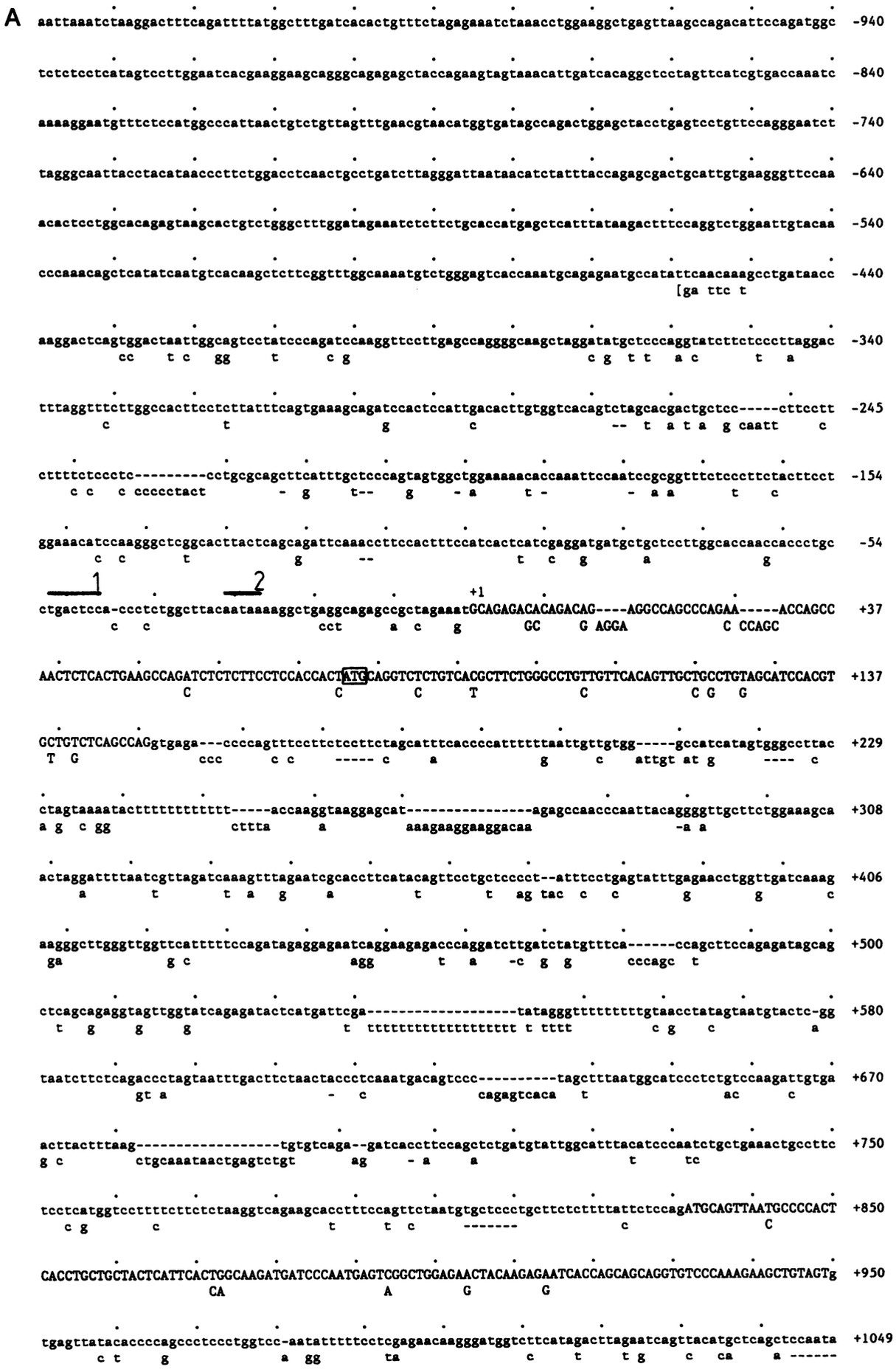
RESULTS

Cloning and characterization of the rat JE gene

To investigate the regulation of JE gene expression in rat cells in more detail we first cloned the gene. A murine JE cDNA probe (a kind gift of Dr. C. Stiles, Boston) cross-hybridizes under stringent conditions to a single fragment of rat genomic DNA (data not shown). A phage-lambda library was constructed from rat DNA, enriched for a 11-kb *EcoR*I-fragment recognized by the JE probe. From the library (6×10^4 plaques) four positive plaques were identified. The inserts from these recombinants comigrate with fragments from rat genomic DNA detected by the murine JE cDNA probe (data not shown) and restriction enzyme mapping indicated that the inserts are identical. The rat JE gene is located on a 4.5-kb *Sph*I-*EcoR*I fragment of the insert (data not shown). After fine mapping of the rat JE gene most of the 4.5-kb fragment was sequenced (Fig. 1A). Comparison with the recently published murine JE gene sequence (15, 33) shows that the genes have the highest degree of similarity in the exon sequences (88%) and in sequences immediately upstream of the gene (88% for the first 160 bp). The rat JE gene consists of three exons of 151, 118 and 521 bp, respectively. The two introns are 680 and 330 bp. Intron sequences are not very conserved between mouse and rat. The splice junctions were confirmed by nuclease S1-mapping (data not shown). Table I shows that the splice junctions in the rat JE gene match with the splice consensus sequences (reviewed in: 34). Potential branch sites are present at approximately 30 bp upstream of the splice acceptor sites.

The high degree of similarity between the rat and the mouse JE gene prompted us to investigate whether this conservation also extends to other species. By using a genomic exon III probe (86% similarity between rat and mouse) at non-stringent conditions we could easily detect a single cross-hybridizing band in hamster DNA. No hybridizing bands could be detected in genomic DNA from human, chimpansee, dog, chicken or fruit fly (data not shown). This result indicates that the JE gene is restricted to rodents, but it does not exclude the possibility that other species have a functionally equivalent gene of a different structure.

The predicted length of the rat JE gene product, like that of the mouse (15), is 148 residues. Fig. 1B compares the rat and murine JE amino acid sequences, which exhibit an overall degree of similarity of 82%. The hydrophilicity plots of the two proteins derived by the Hopp-Woods algorithm are almost identical (data not shown). The amino-terminal half of the protein might be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  ac g            a      t     c     T                        G             G

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G      G                        AC                                G        G     T          C

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 C  C           T   A                       G  TGG        TG         A  T T           G  G

ATCCATTTTTTTATTTCTCTGTGAATCCTGGTTCAACACTTTCAATGTATGAG---------------AGATGAATGGGTAAACTTTGTGTTTGAGAGTC  +1733
     --     CCC            A A                    AGATGAATTTTGTAA                          T

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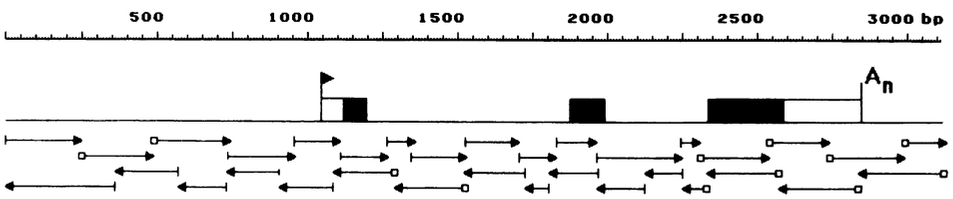

B

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                    20                  40
MQVSVTLLGLLFTVAACSIHVLSQPDAVNAPLTCCYSFTGKMIPMSRLEN
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MQVPVMLLGLLFTVAGWSIHVLAQPDAVNAPLTCCYSFTSKMIPMSRLES

          60                  80                  100
YKRITSSRCPKEAVVFVTKLKREICADPNKEWVQKYIRKLDQNQVRSETT
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YKRITSSRCPKEAVVFVTKLKREVCADPKKEWVQTYIKNLDRNQMRSEPT

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VFYKIASTLRTSAPLNVNLTHKSEANASTLFSTTTSSTSVEVTSMTEN
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TLFKTASALRSSAPLNVKLTRKSEANASTTFSTTTSSTSVGVTSVTVN
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important for its proposed function in intercellular communication, since it is more conserved than the carboxy-terminal half (89% versus 74%). Eight of the ten 'wobble' mutations lie in the amino-terminal half of the protein. Kawahara and colleagues (33) have classified the JE gene as a member of a family of inducible genes encoding small secreted proteins. Cysteine and proline residues at certain positions are a common feature in this family. These residues are conserved between rat and mouse JE. The potential N-linked glycosylation site at 126 is also conserved.

The transcription-initiation site of the JE gene was determined by nuclease S1-mapping (Fig. 2A) and by primer extension (Fig. 2B) using poly(A)$^+$-RNA from different rat cell lines. Both experiments show a major 105 nt and a minor 103 nt band. Comigration of the sequence ladder indicates that the guanine residue at +1 (Fig. 1A) is the major transcription-initiation site. This site conforms to the pyrimidine-purine-pyrimidine rule for initiation sites (35). Position +3 could be a less favoured site.

The 3'-end of the rat JE gene was determined by nuclease S1-mapping using a single-stranded probe from +2093 to +1543. Fig. 2C shows that RNAs from different rat cell lines protect a single band of 255 ± 8 bp. Subsequent experiments using longer probes also show a single protected fragment (data not shown). Since addition of poly(A)-tails frequently occurs at CA-dinucleotides (for review: 36), positions 1800 and/or 1802 are candidate sites for poly(A)-addition. These positions are not preceded by the typical poly(A)-addition signal. However, three conserved hexanucleotides, which all contain one mismatch to the canonical sequence ATTAAA, are present around +1780 (Fig. 1A). This nested set of weak signals might function as an efficient poly(A)-addition signal. On the other hand, AATTAA at +1774 could be sufficient. This hexanucleotide has been recognized as an alternative to the canonical site (36). Positions +1816 to +1825 form a GT-rich sequence, which frequently follows poly(A)-addition sites (36).

Regulation of JE gene expression

Nuclear run-on experiments have indicated that induction of the JE gene by various growth factors occurs at the level of transcription (unpublished results; 6, 37). This predicts that the JE promoter is able to confer inducibility to a reporter gene. To test this we performed a serum-stimulation assay with NIH 3T3 cells stably transfected with the bacterial chloramphenicol acetyltransferase (CAT) gene under control of the rat JE gene promoter. Cultures were made quiescent by incubation in medium containing 0.5% serum. Subsequently, cells were stimulated with 20% fetal calf serum. RNA was isolated at different time points and CAT RNA levels were analysed by an RNAse-protection assay (Fig. 3). In cell lines transfected with the JE-CAT construct, expression of CAT RNA is induced 90 min after serum stimulation (lanes 3 and 7). After 4½ hours CAT RNA has returned to lower levels. This can be explained by the short half-life of CAT RNA, which like that of JE mRNA, is about 90 min (6, 17; I. Offringa-Laird, unpublished observations). This suggests that in these experiments the induction of the JE promoter was transient, which is also known to be the case for the endogenous JE gene (2, 6, 17). In the RSV-CAT cell line (G2.4) CAT RNA is also induced by serum (lanes 11 and 12). However, this induction is not very strong and has different kinetics than is observed in the JE-CAT cell lines. Other RSV-CAT cell lines showed a similar weak induction (data not shown). This indicates that the RSV-LTR responds weakly to serum. The results of Fig. 3 show that the JE promoter can render a reporter gene serum-inducible. This implies that induction of JE expression by serum is mediated by an activation of the promoter.

To delineate the important region(s) of the JE promoter we constructed a panel of plasmids containing different JE promoter fragments in front of the CAT gene. All JE promoter constructs ended at +59 and were inserted by a 12-bp linker into the *Hin*dIII site of pBA-CAT (25). Basal activity of the constructs was tested by transient expression in proliferating Rat-1 cells. Plasmids were cotransfected with 1 μg pRSV-hGH plasmid and transfection efficiencies were normalized by taking into account the levels of secreted human growth hormone. Deletion of sequences upto −70 was found to have no effect (Fig. 4). All constructs gave approximately the same CAT activity, except JE-38, -24, -543E and -543B. This implies that 70 bp upstream of the cap site are sufficient for the activity of the promoter. Deletion from −70 to −38 gave a five-fold reduction in activity, indicating that this region is important. Removal of the putative TATA-box had no effect on the promoter activity (compare JE-38 and JE-24). Deletion from −24 to +4 reduced the activity further to background levels (data not shown), indicating that this region might have some weak activity. However, the −70/−38 region seems to contain the most important element(s) for basal activity of the promoter.

The −70/−38 region harbors the sequence TGACTCC (−52/−46), which is related to the palindromic AP-1-binding site consensus TGACTCA (38). For convenience we will refer to the −52/−46 sequence as the JE AP-1 site. The plasmids JE-543E and -543B are derived from JE-543 and contain mutations in the JE AP-1 site, that result in TGA*AT*CC and TG*GA*TCC, respectively. The JE-543B construct shows a two-

TABLE I. SPLICE SITES

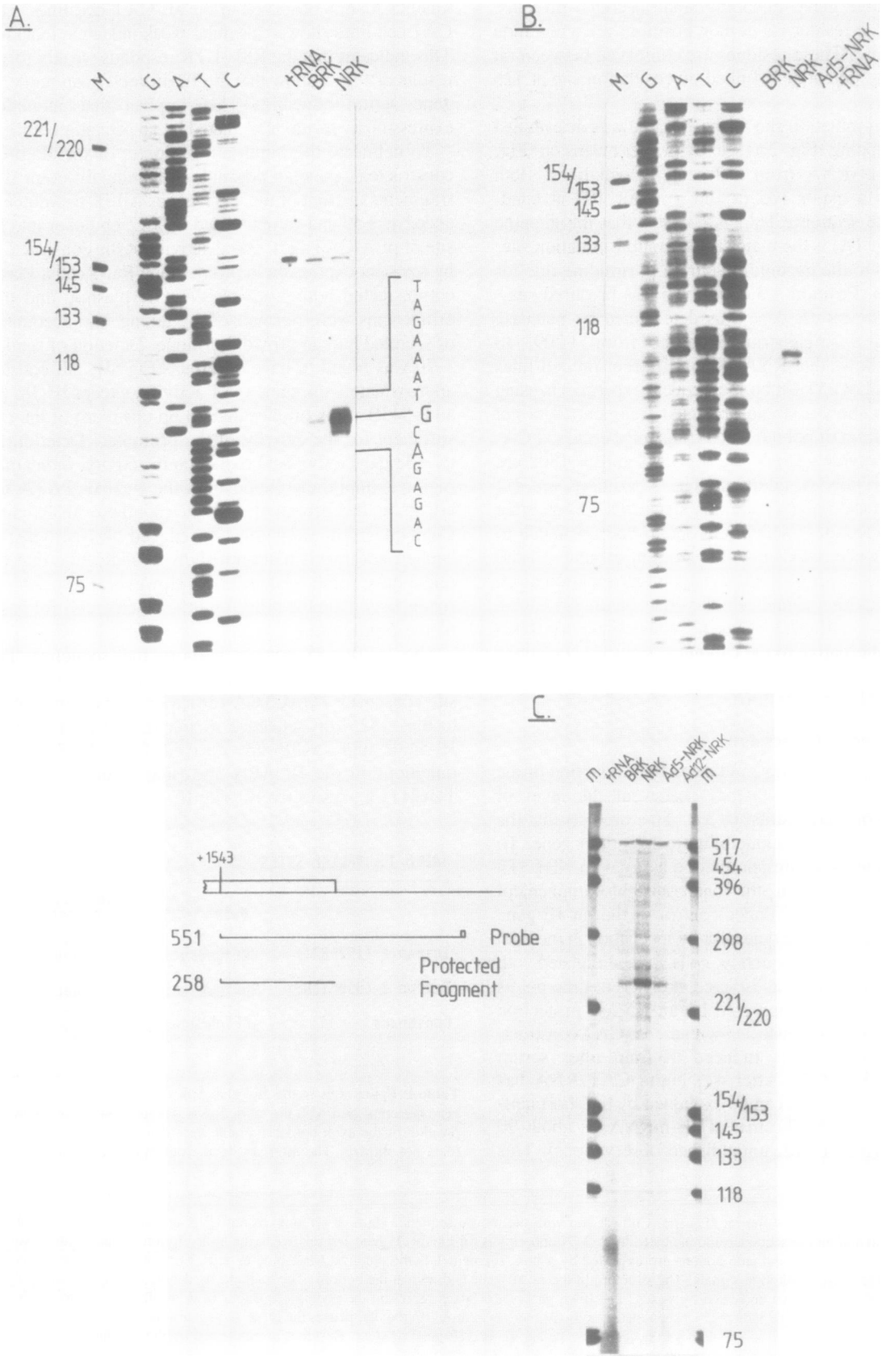
	donor	potential branch	acceptor
Intron 1 (152-831)	CAG↓gugaga	797 * cuaau	y_{14}acag↓A
Intron 2 (950-1289)	AGU↓gugagu	1263 * cuaac	y_{10}ay$_{5}$cag↓A
Consensus	$^{C}_{A}$AG↓gu$^{g}_{a}$agu	yu$^{a}_{g}$*ac	y_{11}ncag↓G

Table I. Splice sites in the JE gene. Splice donor and acceptor sites of the JE gene are compared with the consensus sequences (34). The arrows indicate the position of the exon borders, which were determined by nuclease S1-mapping (data not shown). The asterisks denote potential branch sites.

Figure 1A. Structure and sequence of the rat JE gene. Comparison with the mouse sequence starts at ([) and ends at (]). Gaps are introduced to obtain maximal similarity between the rat and mouse sequence and indicated by (−). Numbering is for the rat JE gene and relative to the transcription-initiation site. Exonic sequences are in capitals. The translation start and stop codons are indicated by a box. Element 1 is the putative AP-1 binding site. Element 2 is the presumed TATA-box. Overlined are the possible poly(A)-addition signals. The structure of the rat JE gene is drawn at the bottom. The flag and A_n denote the cap site and poly(A)-addition sites, respectively. The three exons are indicated by boxes. The filled part of the boxes represent the coding sequence. Underneath, the arrows show the sequence strategy. Arrows with an open rectangle indicate that sequence-specific primers were used. In the other cases the DNA sequence was determined from deletion clones.
Figure 1B. Comparison of the deduced amino acid sequences of the rat and mouse JE gene product. Residues are indicated by the one-letter code for amino acids. The asterisks denote the possible site for N-linked glycosylation.

fold reduction in activity, but the -543E mutations almost completely abolish promoter activity (Fig. 4). Together with the evidence from the deletion clones this mutation analysis suggests that this putative AP-1 site is an important element of the JE promoter.

Transcription of the JE gene can also be induced by the phorbol



ester TPA (12-O-tetradecanoyl-phorbol-13-acetate; 6, 14, 17). An exception is found for Rat-1 cells in which the endogenous JE gene is not TPA-inducible (H. van Doorninck and H.Th.M. Timmers, unpublished results). In order to define the TPA-responsive element (TRE) of the JE promoter the various CAT constructs were tested for their transient expression in HeLa TK^- cells, which are frequently used in such experiments. TPA induction of the JE promoter is about four- to five-fold (Fig. 5). Removal of sequences upto −141 has no effect on the relative induction by TPA. In this experiment JE-328 shows an elevated induction, but this was not observed in other experiments. Deletion of sequences between −141 and −88 abolishes the TPA-inducibility. The JE-88 and JE-70 constructs have a low basal activity, but show no induction by TPA. No activity can

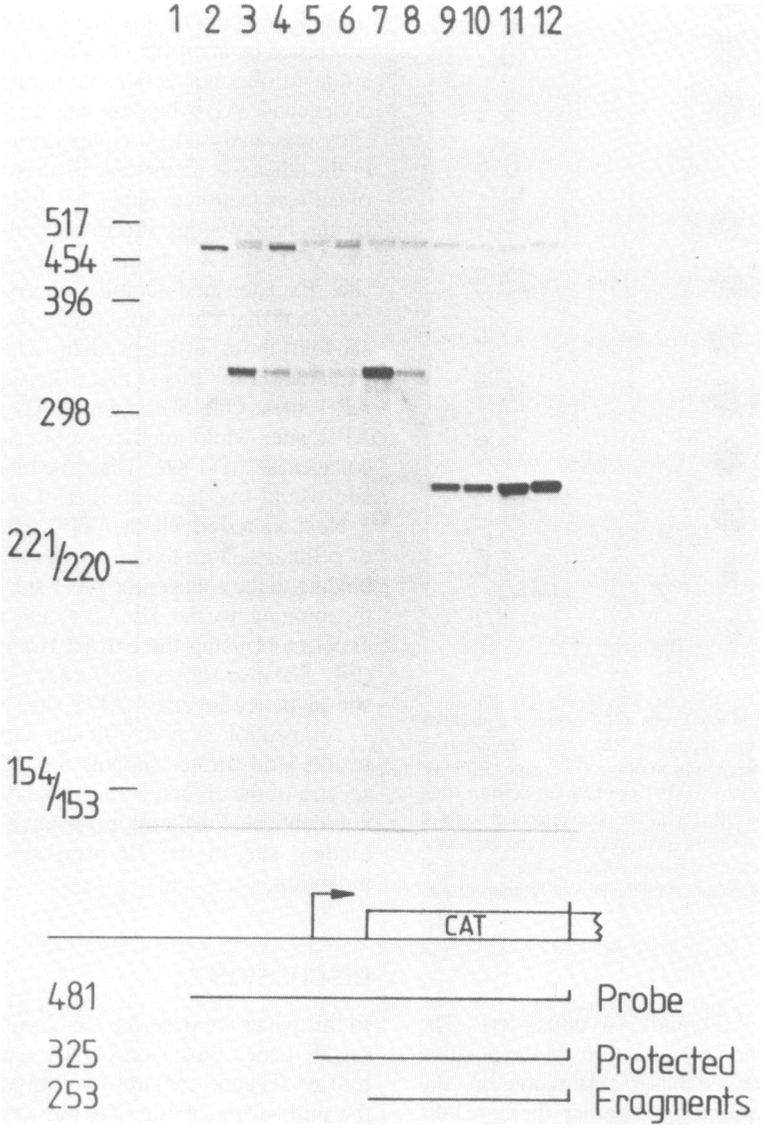


Figure 3. The JE promoter confers serum inducibility to a reporter gene. RNA from NIH 3T3 cell lines stably transfected with CAT plasmids was analysed by an RNAse protection assay. The uniformly-labelled probe of 481 nt is complementary to the JE-CAT plasmid from position −177 of the JE promoter to the *EcoR*I site in the CAT gene. Twenty μg cytoplasmic RNA of two independent JE promoter-CAT cell lines (lanes 1−4 and 5−8) or a RSV-CAT cell line (lane 9−12) was used. RNA was from proliferating cells (lanes 1, 5, 9), from quiescent cells (lanes 2, 6, 10) or from cells subsequently stimulated by 20% serum for 90 min (lanes 3, 7, 11) or for 4½ h (lanes 4, 8, 12). Hybridization was at 36°C. Positions of comigrated marker fragments are indicated.

Figure 2. Determination of the transcription-initiation and poly(A)-addition site. The cap site was determined by nuclease S1-mapping and by primer extension. Panel A shows the fragments protected from nuclease S1-digestion. A single-stranded radioactively labelled probe spanning positions +105 to −50 was prepared by extending an oligonucleotide primer (complementary to +105/+88) on an appropriate M13 clone. Twenty μg of cytoplasmic RNA from primary baby rat kidney (BRK) cells or NRK 49F cells were used for hybridization at 52°C. Transfer RNA from yeast served as a control. The same oligonucleotide primer was used for the DNA sequence ladder, which was comigrated on the 5% polyacrylamide/urea gel. Labelled marker fragments were loaded in lane M and fragment lengths are indicated. Panel B shows the reverse transcriptase extension products of the 5'-labelled !oligonucleotide primer. Twenty μg of oligo(dT)-selected RNA from BRK, NRK 49F or Ad5 E1-transformed NRK cells were used for extension. The sequence ladder from the same labelled oligonucleotide and marker fragments were comigrated on the same gel. The end of the JE mRNA was determined by nuclease S1-mapping (panel C). A single-stranded radioactively labelled probe was prepared by extending an oligonucleotide complementary to position + 2093 to +2076 on the appropriate M13 clone. The probe spans position +2093 to +1543 and was hybridized to RNA from indicated rat cells at 42°C. Protected fragments were separated in a 5% polyacrylamide/urea gel. In lanes m the above mentioned marker fragments were comigrated.

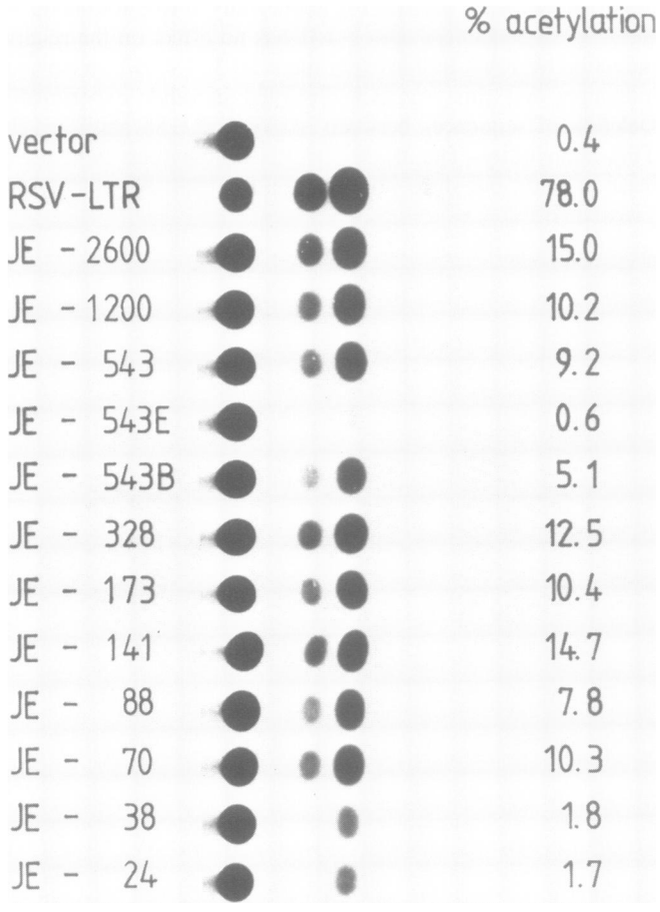


Figure 4. Analysis of the rat JE promoter in Rat-1 cells. CAT expression plasmids under control of various promoters were transfected into Rat-1 cells. The numbers indicate the length of JE promoter sequences in front of the CAT gene. JE-543E and JE-543B contain mutations in the putative AP-1 site and are derived from JE-543. After normalization of transfection efficiencies via measurement of secreted human growth hormone CAT activity was determined, quantitated and expressed as the percentage converted into acetylated chloramphenicol. About 200 μg of protein was used to assay the CAT activity, except from the RSV-LTR transfection, of which 50 μg was used.

be observed with the JE-38, −24 and +4 constructs. The JE-543E and 543B constructs, carrying mutations in the putative AP-1 binding site, show a reduced basal expression but the induction by TPA remains unaffected. Together these results indicate that the TRE of the JE promoter is located between −141 and −88. The JE AP-1 site does not appear to be important for TPA induction.

Transcription of the JE gene can be repressed by the adenovirus E1A gene (17). Therefore we tested whether plasmids expressing E1A can inhibit expression of the JE promoter-CAT constructs in cotransfection experiments. It has recently been found that TPA induction of the collagenase promoter can be inhibited by E1A (R. Offringa, S. Gebel, H.Th.M. Timmers, A. Smits, R. Zwart, B. Stein, J.L. Bos, A. van der Eb and P. Herrlich, submitted). This promoter was included as a control in the experiment of Fig. 6. Whereas a strong inhibition of the collagenase promoter was observed, E1A did not inhibit the JE promoter. The failure of E1A to inhibit JE-CAT in transient assays was also observed in several other cell lines (Rat-1, NRK 49F, L929). This result was unexpected and could not be explained. At least, the data of Fig. 6 suggest that E1A utilizes different mechanisms to repress the collagenase and the JE gene.

Binding of cellular factors to the −52/−46 element

The putative AP-1 binding site, TGACTCC at −52, is important for basal activity but not for TPA induction of the JE promoter (Figs. 4 and 5). The AP-1 consensus sequence, TGACTCA, is required for the response of the collagenase promoter to TPA (19). An explanation for the functional difference between these sequences would be that they bind different cellular factors. This was tested by an *in vitro* binding assay using radiolabelled double-stranded oligonucleotides containing either the JE AP-1 or the collagenase AP-1 binding site as probes. Affinity for the AP-1 sites was analysed by competition with either the homologous or the other site. In the case of an extract from Rat-1 cells, binding of cellular factors to either AP-1 site is inhibited more efficiently by the homologous site than by the other (Fig. 7). Binding to the AP-1 sites is not competed by the unrelated H2TF1-binding site. Excision and scintillation counting of the retarded bands indicated that competition with the homologous site is three to six-fold more efficient than with the other AP-1 site. An explanation for this is that different cellular factors bind to the AP-1 sites. One factor would have a higher affinity for the JE AP-1 site, while another would have a higher affinity for the collagenase AP-1 site. Thus, the binding spectrum of the JE AP-1 site would overlap with that of the collagenase AP-1 site.

Next we tested whether TPA treatment influences the binding of cellular factors to the various AP-1 sites. Fig. 8A shows that binding to the collagenase AP-1 site is increased by TPA, whereas the binding to the JE AP-1 site is not. A cross-competition experiment using the extract from TPA-stimulated HeLa cells (Fig. 8B) also shows a difference in competition of the JE AP-1 site or the collagenase AP-1 site. Similar to the results of Fig. 7, competition is more efficient with the homologous site. These results lend further support for the hypothesis that the binding spectra of the JE and collagenase AP-1 sites are overlapping but not identical. Furthermore, it can explain why the putative AP-1 binding site in the JE promoter does not respond to TPA induction.

DISCUSSION

In this paper we describe the cloning and characterization of the rat JE gene. Based on cDNA data it was concluded that the murine JE gene contains two poly(A)-addition sites (15, 33). In the numbering of Fig. 1A the first of those sites is at +1590 and the other at +1802. In rat cells we can detect only JE RNA ending at the +1800/+1802 site and no RNA ending around +1590 (Fig. 2C). This result is supported by RNA blotting analysis, which does not show JE RNA shorter than 800 nucleotides (unpublished observations). Furthermore, the putative poly(A)-addition signals for the +1590 site (at +1566 and at +1573) are not conserved in the rat sequence. This is probably the cause for the exclusive use of the +1800/+1802 site in rat cells.

There is some controversy about the precise 5′ end of the murine JE gene. Rollins and colleagues (15) have determined position +3 (in the numbering of Fig. 1A) as the transcription-initiation site, while Kawahara (33) have mapped it at −3. Using two approaches we show that the guanine residue at +1 is the major transcription-initiation site for rat JE RNA (Figs. 2A and 2B). The adenine residue at +3 might be a minor site. No

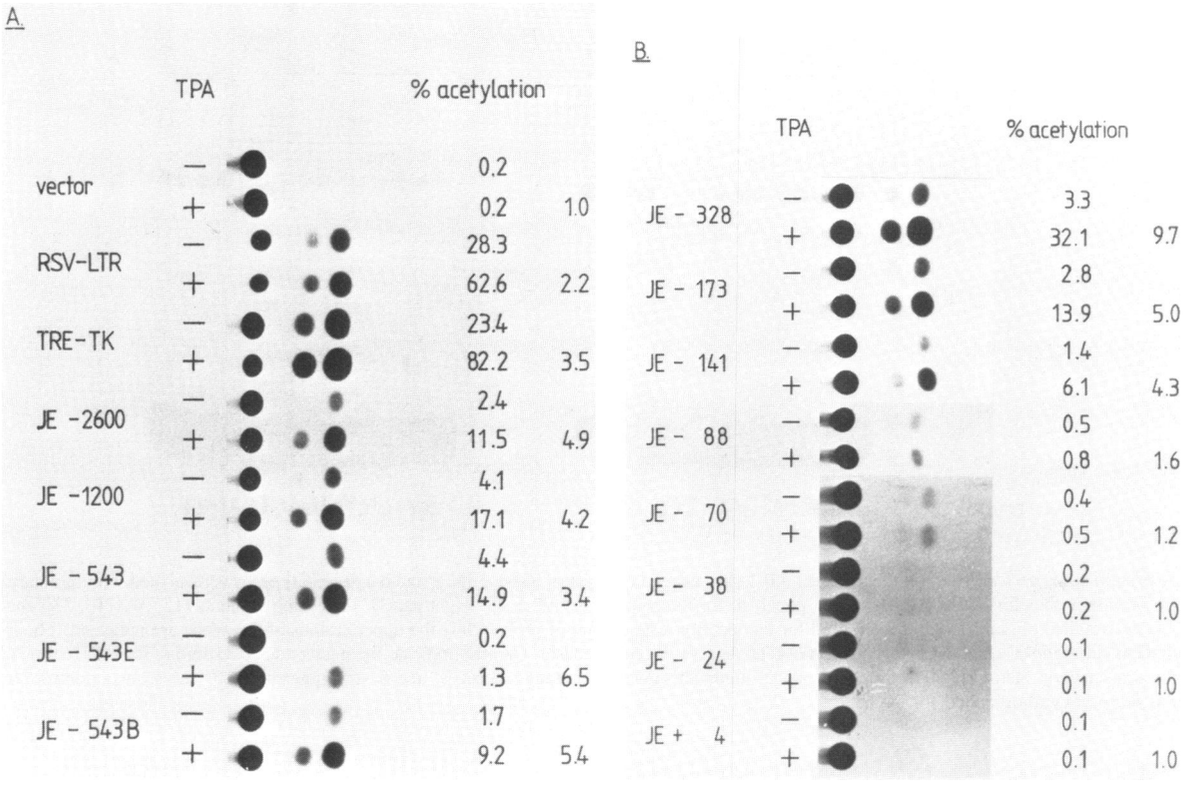


Figure 5. Determination of promoter sequences required for induction by TPA. Different CAT expression plasmids were transfected into HeLa TK$^-$ cells and immediately after transfection 100 ng/ml TPA was added, where indicated. CAT activity was quantitated as in Fig. 4. The last column expresses the TPA induction factor. The TRE-TK promoter plasmid carries a single copy of the TPA-responsive element of the collagenase promoter, TGACTCA, and acts as a control for TPA induction. About 200 μg of lysate was used to assay CAT activity, except from the RSV-LTR and TRE-TK transfections, of which 20 μg was used.

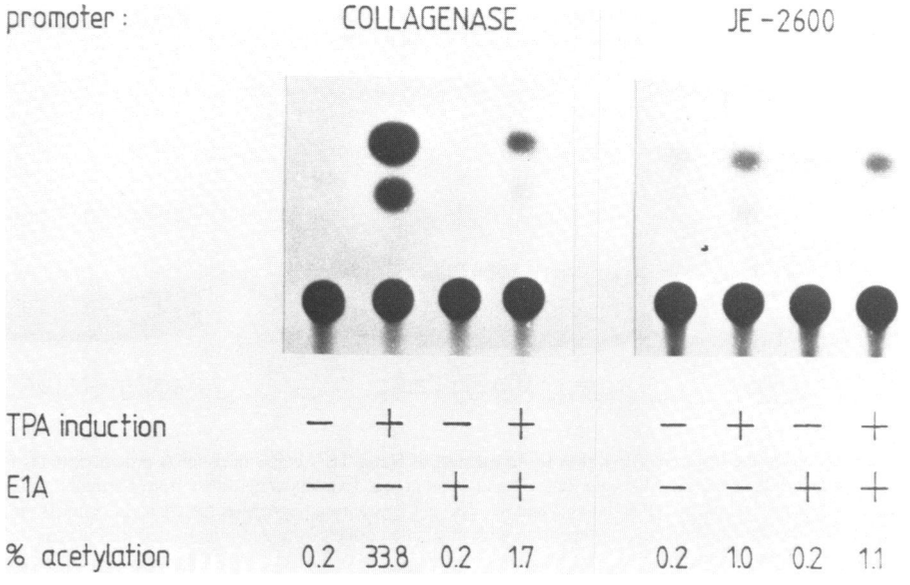


Figure 6. Influence of cotransfected E1A expression plasmids on JE promoter activity. HeLa TK$^-$ cells were transfected with 4 μg promoter-CAT plasmid and, where indicated, 16 μg RSV-E1A plasmid was cotransfected. About 5 μg and 150 μg lysate was used from the collagenase and the JE transfections, respectively.

transcripts starting at −3 could be detected. We note that only the position +1 conforms to the Py-Pu-Py consensus sequence for initiation sites (35). However, this rule is rather loose and knows numerous exceptions.

Regulation of JE expression occurs at the transcriptional level (6, 17, 37) and Fig. 3 indicates that in this process JE promoter sequences play an important role. Transient expression assays show that two elements, −70/−38 and −24/+4, are required

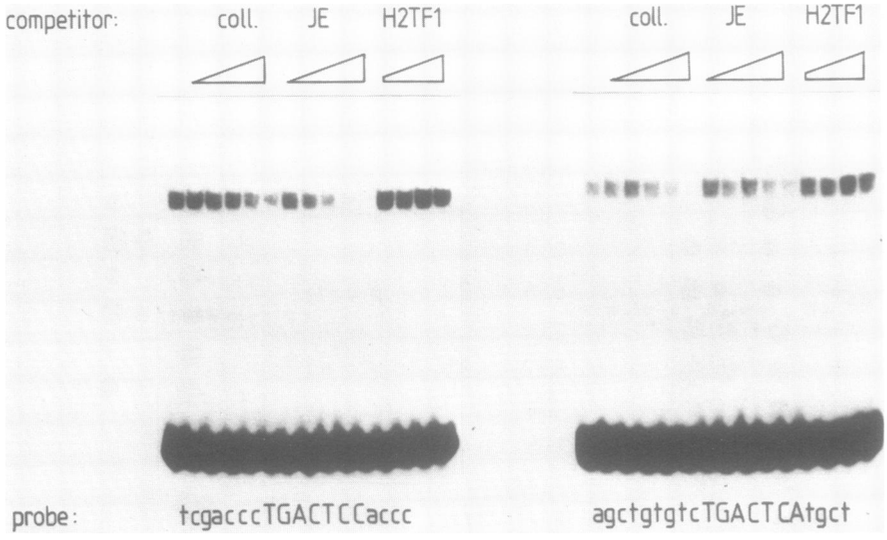


Figure 7. DNA-protein complex formation on the different AP-1 sites using an extract of Rat-1 cells. Protein extract (10 μg) was incubated with radiolabelled double-stranded oligonucleotide probes for the JE AP-1 site (left panel) or for the collagenase AP-1 site (right panel). Increasing amounts (12, 37, 110, 330 and 1000 fmol) of unlabelled double-stranded oligonucleotides were used for competition with 3 fmol of probe. Only the upper strands of the probe are indicated. The lower strands are 5′-gatcgggtGGAGTCAggg-3′ (JE) and 5′-agctagcaTGAGTCAgacac-3′ (collagenase). Capitals indicate the nucleotides constituting the AP-1 site. A binding site for the unrelated transcription factor H2TF1 composed of the oligonucleotides 5′-agctggggattcccc-3′ and 5′-agctggggaatcccc-3′ served as a control (from 37 fmol to 1 pmol). Gels were autoradiographed for 20 h.

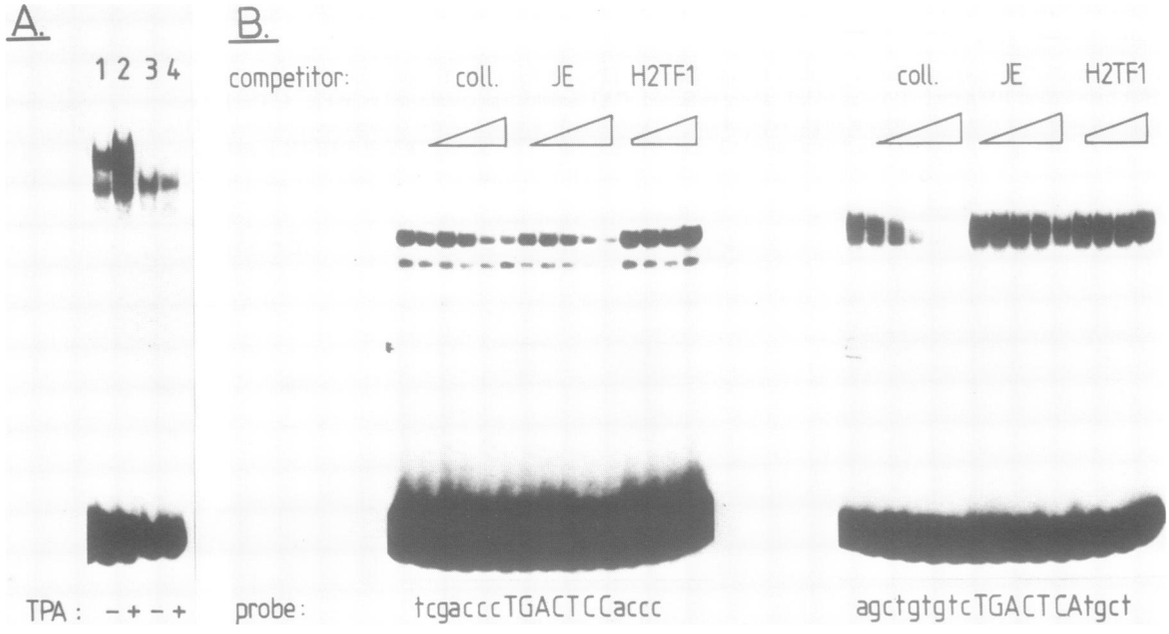


Figure 8. DNA-protein complex formation on the different AP-1 sites using extracts of HeLa TK$^-$ cells. In panel A protein extracts (10 μg) were incubated with either the collagenase AP-1 site (lanes 1 and 2) or the JE AP-1 site (lanes 3 and 4) as probe. Extracts were either from normally growing HeLa cells (lanes 1 and 3) or from HeLa cells stimulated for 6 h with 100 ng/ml TPA (lanes 2 and 4). The gel was autoradiographed for 24 h. In panel B the extract (10 μg) from TPA-stimulated HeLa cells was incubated with the indicated probes. Competition with unlabelled double-stranded oligonucleotides was as in Fig. 7. The right panel was autoradiographed for four days and the left panel for 24 h.

for basal activity of the JE promoter in Rat-1 cells. It is interesting to note that the rat and murine sequences are quite similar in the −70/−38 region, but vary in the −24/+4 region. The conserved TATA-like sequence, AATAA at −30, apparently has no effect on promoter activity, while the −24/+4 region has a weak activity. This region has no obvious similarity to the recently identified Initiator element (39), which overlaps with the cap site of the terminal deoxynucleotidyltransferase gene and is sufficient for its basal promoter activity. The −70/−38 region carries the major activity of the JE promoter. This region contains the TGACTCC element at −52 which is similar to the AP-1 consensus binding site, TGACTCA (38). The functional importance of the element at −52 is stressed by mutation analysis. Binding of a cellular factor to the putative JE AP-1 binding site can be competed with an AP-1 consensus site. However, competition is less efficient than with the homologous site.

Overlapping specificities for different binding sites could be a recurrent theme for DNA-binding factors. Each CCAAT-binding factor binds to a subset of CCAAT boxes only, but the specificities of CCAAT factors form overlapping sets (reviewed in: 40).

Several agents like serum, poly(I-C), EGF and TPA can induce JE mRNA expression. We have tested whether serum can induce the JE promoter in transient transfection of Rat-1 cells and could not detect a specific induction (unpublished results). In contrast, the endogenous JE gene is induced by serum (H. van Doorninck and H.Th.M. Timmers) and stably integrated JE-CAT genes are serum-responsive (Fig. 3). The reason for this discrepancy is unclear, but chromatin structure might be important for the response of JE to serum.

We have previously shown that transcription of the JE gene is severely repressed in cells transformed by adenovirus E1A (17). Transient E1A-cotransfection experiments show no inhibition of JE promoter activity. Similarly, activity of JE-CAT constructs is not inhibited when the constructs are transiently introduced into E1A-expressing cells (unpublished results). Several explanations can be offered for the lack of inhibition by E1A in these experiments. A trivial one is that the target for E1A repression is not located on the 2.6-kb promoter fragment tested. Secondly, E1A might induce a low-abundant transcriptional repressor, which is competed out in transient transfections. Thirdly, chromatin structure could be important for the E1A-mediated repression of the JE gene. And fourthly, the repression by E1A might be indirect and not measurable in short term-assays. At present we cannot discriminate between these possibilities.

Transcription of the JE gene can be induced by the phorbol ester TPA (37). We could observe induction of the JE promoter in transient assays (Fig. 5). One of the promoter elements known to respond to TPA is the binding site for NF-kB (41). A potential NF-kB site was noted at −154 in the murine JE sequence (42). This element is not conserved in the rat JE promoter. Moreover, deletion analysis of the JE promoter indicates that the −141/−88 region is required for the response to TPA (Fig. 5). This region does not contain any of the elements known to respond to TPA. A further analysis of the −141/−88 region is required to identify the target for TPA in the JE promoter.

The AP-1 binding site in the collagenase promoter mediates the TPA response of this promoter (19). Surprisingly, the related JE AP-1 site at −52/−46 appears not to be not involved in the response to TPA. Figs. 7A and 7B indicate that *in vitro* the collagenase AP-1 and the JE AP-1 site have a different specificity for cellular factors, but that their binding spectra overlap. It has been shown that transcription factor AP-1 consists of several components (38) and binds as a dimer (reviewed in: 12). Heterodimers composed of a member of the *fos* family and of the *jun* family bind very avidly to the consensus AP-1 site (which is identical to the collagenase AP-1 site), while *jun* homodimers bind less efficiently. However, it was shown that bacterially produced v-*jun* efficiently binds (presumably as a homodimer) to the TGACTCG element in the hamster histone H3.2 promoter, whereas purified AP-1 from HeLa cells does not bind (43). It is interesting to note that like the JE AP-1 site, the histone element deviates from the consensus AP-1 site at the seventh position. The observation that the JE AP-1 and the collagenase AP-1 sites bind different types of AP-1 (Figs. 7A and 7B) can explain the different behaviour of these sites *in vivo*. If this explanation is correct, only some and not all AP-1 dimers are capable of mediating TPA induction. AP-1 proteins specific for the JE AP-1 site should be constitutively active and could, thereby, maintain the basal activity of the JE promoter. Another possibility is that two sequences (e.g. the −141/−88 and the JE AP-1 sequences) act synergistically to mediate the response to TPA. Although the mutation analysis indicates the JE AP-1 site is only important for the basal expression and not for the induction by TPA (Fig. 4 and 5), it cannot be excluded that the residual activity of the mutated AP-1 site is sufficient to cooperate with the −141/−88 region. We are currently investigating these possibilities.

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