MOLECULAR AND CELLULAR BIOLOGY, Oct. 1989, p. 4265–4271
0270-7306/89/104265-07$02.00/0

Vol. 9, No. 10

Novel Regulation of Transcription Initiation of the Peptide IX Gene of Adenovirus 2

TAKASHI MATSUI

Department of Molecular Biology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807, Japan

Received 13 March 1989/Accepted 20 June 1989

***cis*-Acting elements involved in transcription of the peptide IX (pIX) gene of adenovirus 2 were identified by using in vivo transient expression assays and two in vitro transcription systems. Deletion of either the sequence between positions −45 and −70 or the TATA box abolished the initiation of pIX gene transcription in vivo and transcription with HeLa cell nuclear extracts in vitro. These results initially suggested the presence of a positive factor acting on the upstream element. However, when proteins in the nuclear extract were fractionated by column chromatography and analyzed by reconstitution of transcription in vitro, it was found that a certain fraction could direct TATA box-dependent transcription initiation even in the absence of the upstream element. Furthermore, activity inhibiting TATA box-dependent transcription was found in the nuclear extract. In contrast, inhibition of TATA box-dependent transcription was suppressed by deletion of a downstream sequence between positions +33 and +122. These results indicate that the TATA box of the pIX gene by itself has the ability to direct initiation of constitutive transcription but that the function of this element is under negative control by a repressor acting on a downstream sequence. Thus, the upstream element of the pIX gene appears to have a novel function: suppression of the transcriptional repression exerted by a downstream sequence, leading to a net transcription activation. Possible mechanisms for transcription initiation of pIX DNA are discussed.**

The promoter region of a eucaryotic gene consists of several sequence elements usually localized upstream of the transcription initiation site (cap site). The TATA box, the element nearest the cap site, is found in most eucaryotic protein-coding genes. A specific factor, called TFIID, has been identified as binding to the TATA box (24). Its consensus in sequence and position (30 base pairs upstream of the cap site) suggests that this factor may be involved in the regulation of all genes containing a TATA box. The elements adjacent to the TATA box (the upstream elements) are more varied than the TATA box in sequence and position. Several factors specific for the upstream elements, which stimulate transcription initiation directed by the TATA box, have been identified (for reviews see references 4, 16, and 20). It has recently been demonstrated that transcription initiation is determined primarily by direct interaction between TFIID and the upstream element-binding factor (8, 10, 11). These two elements are thought to be the core of eucaryotic mRNA gene promoters. In most eucaryotic genes, including viral genes, promoters are controlled by other *cis*-acting elements, the enhancers. The enhancers are thought to be involved in tissue- or cell-specific regulation of transcription and are *cis*-acting activators that function independently of orientation and distance from the coding sequences of the genes. Several models to explain the mechanism of enhancer-activated transcription have been proposed (for reviews, see references 1 and 25). In addition to containing the positive *cis*-acting elements, many eucaryotic genes are known to contain negative *cis*-acting elements (5, 9, 13, 14, 21, 23, 28; reviewed in references 1 and 15). The importance of negative regulation in the transcription of eucaryotic genes is becoming increasingly apparent. The existence of repressor molecules was suggested originally by use of cell fusions between differentiated cell types and fibroblasts (2, 7, 12, 22, 30). Several lines of evidence indicate that negative regulation of transcription is involved in tissue- or cell-specific and inducible gene expression. For example, the inducible enhancer element of the β-interferon gene consists of two parts, the 5′ part as a constitutive transcription element and the 3′ part as a negative element (6, 32). A factor bound to the negative element is found to be present in uninduced cells. Furthermore, induction leads to dissociation of the factor from the element and to the binding of a different factor to a different region within the enhancer (31). Thus, the on-off mechanism of transcription appears to be controlled by the differential appearance of *trans*-acting positive and negative factors.

I and others have previously reported that transcription of the adenovirus 2 peptide IX (pIX) gene is activated by DNA replication in a transient expression assay (19, 27). Transcription of the pIX gene occurred only on DNA molecules that had been replicated in transfected HeLa cells. This observation led me to speculate that pIX DNA introduced into cells is primarily repressed and that an active transcription complex is constructed only after DNA replication. By analysis of the fate of pIX DNA in transfected cells, supercoiled DNA was found to be quickly relaxed in the cells. After replicating, however, the relaxed DNA became supercoiled. This change in DNA form was dependent on DNA replication. Furthermore, supercoiling of pIX DNA seemed to result from the formation of pIX DNA into nucleosome structures (unpublished observation). Since the adenovirus E1a gene was active irrespective of replication of its DNA, it is not clear whether the formation of pIX DNA into nucleosome structures is involved directly in the activation of transcription of the pIX gene via DNA replication. To examine how transcription of the pIX gene is activated by replication of its DNA, it is essential first to identify the sequence elements that are involved in the initiation of pIX gene transcription. In this study, it is shown by use of in vivo and in vitro transcription systems that the level of transcription of the pIX gene is determined by three *cis*-acting

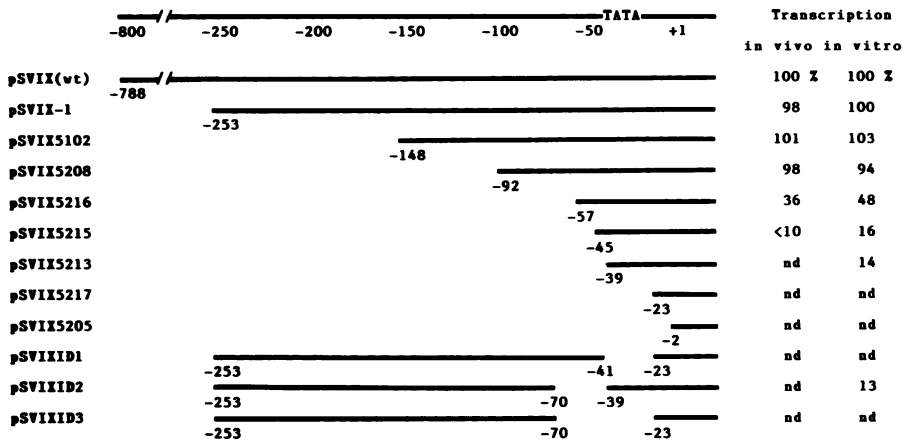

FIG. 1. Transcription of the pIX gene from various mutant DNAs. Transfection of DNA and in vitro RNA synthesis were performed as described in Materials and Methods. The amount of pIX gene transcript was determined by densitometry of the autoradiograms and was normalized by estimation of the amounts of cotransfected E1a gene transcript and simian virus 40 T-antigen transcript. The relative amount of transcript from each DNA was estimated by calculating the amount of transcript from pSVIX DNA (100%). ND, Not detected.

elements: the TATA box, the upstream element, and the downstream element. The first two elements were found to be positive elements, as determined by the inactivation of transcription that resulted from their deletion. The downstream element acted in a negative manner in the absence of the upstream element. In addition, a *trans*-acting factor(s) responsible for specific inhibition of transcription of pIX DNA was found to be present in HeLa cells. Thus, the upstream element of pIX DNA appeared to perform a novel function: activating transcription by suppressing transcriptional repression.

MATERIALS AND METHODS

Cell culture and transfection. Maintenance of HeLa cells and DNA-mediated transfection were carried out as described previously (19) except that the cells were exposed to a DNA–DEAE-dextran solution for 60 min.

Plasmid construction. pSVIX plasmid DNA (19; Fig. 1) was used as the wild type. To make a series of 5′-deletion mutants, pSVIX DNA was first digested with *Bgl*II (−253) and then with BAL 31. After ligation of the *Bam*HI linker (CGGATCCG) and extensive digestion with *Bam*HI, the DNA fragment purified through agarose gel electrophoresis was self-ligated and used for transformation of *Escherichia coli* HB101. pSVIX-1 DNA (Fig. 1) was digested with *Sac*I and then with BAL 31 to make a series of 3′-deletion mutants. Ligation of the *Bam*HI linker, recovery of the DNA fragment, and transformation were carried out as described above. To construct mutant pIX DNA deleted of both upstream and downstream regions, pSVIX5215 (Fig. 1) was digested with BAL 31 at the *Sac*I site (+58). To construct internal deletion (ID) mutant clones, appropriate 5′- and 3′-deletion clones were digested with *Bam*HI and *Eco*RI, and the digests were separated on a 1% agarose gel. The fragments containing the 5′ and 3′ parts of pIX DNA were ligated.

Preparation of HeLa cell extracts and fractionation of proteins. Nuclear, whole-cell, and S100 extracts of HeLa cells were prepared as described previously (3, 17, 29). Nuclear extracts were applied on a phosphocellulose (P-11) column equilibrated with buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 25% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol) containing 0.1 M KCl. The column was washed with the same buffer, and the bound protein was eluted stepwise with 2 column volumes of buffer A containing 0.3, 0.6, and 1.0 M KCl. In some experiments, nuclear extracts were applied on a DEAE-cellulose column equilibrated with buffer A containing 0.1 M KCl. The column was washed with the same buffer, and the bound protein was eluted with buffer A containing 0.4 M KCl. The appropriate flowthrough and step-eluted fractions were pooled on the basis of A_{280} and dialyzed for 4 h against buffer A containing 0.1 M KCl.

Analysis of in vivo and in vitro RNA by P1 nuclease mapping. Total cytoplasmic RNA was prepared and analyzed by P1 nuclease protection mapping to determine the transcription levels of mutant pIX gene clones as described previously (19). In vitro RNA synthesis and analysis were performed as described previously (18).

RESULTS

Structure of the pIX gene promoter. When pSVIX and pSVIX-1 DNAs, containing 753 and 253 base pairs, respectively, of upstream flanking sequences were transfected into HeLa cells, almost identical levels of transcription of the pIX gene were observed, indicating that the sequence elements necessary for maximal transcription lie at a certain distance downstream from −253. To determine the regulatory sequences for the pIX gene, a series of 5′-deletion mutant DNAs was constructed by removing the downstream sequences from the *Bgl*II site at −253 in pSVIX DNA. Each deletion clone was then cotransfected with pHindG DNA containing the adenovirus 2 E1A gene. The levels of transcription of these deletion DNAs were analyzed by P1 nuclease mapping of total cytoplasmic RNA from the transfectants. In these experiments, the E1a gene transcript was used as an internal standard for quantifying the pIX gene transcript.

Deletion of the sequences upstream of −92 did not change the transcription levels in comparison with that of the wild-type DNA (Fig. 1). However, when the deletion reached −57, transcription decreased to about 40% of the wild-type level. Further deletion to −45 caused almost complete inactivation of pIX gene transcription. These results indicate that the sequence between −92 and −45 is necessary for the initiation of pIX gene transcription. Next,

to analyze whether a typical TATA box found at −30 is involved in the initiation of transcription, three ID mutants were constructed and the levels of transcription were analyzed. Clone ID1 has a deletion in the sequence from −23 to −41 and thus is missing the TATA box. Clone ID2 has a deletion in the sequence from −40 to −70. Clone ID3 has a deletion in both the upstream and TATA box regions, from −23 to −70. None of the three ID clone DNAs were transcribed significantly. Therefore, both the upstream sequence between −45 and −70 and the TATA box were required for maximal transcription of the pIX gene in transient expression assays. Either of these two sequence elements alone did not direct transcription of the pIX gene. The effect of deletion of the downstream region was also analyzed in vivo; no region was found to significantly affect the level of transcription of pIX DNA (see below).

Next, the effects of the upstream elements were examined in vitro. The deletion mutants used in the in vivo assays were assayed in an in vitro transcription system supplemented with nuclear extracts. To avoid fluctuation in the efficiency of transcription reconstitution, the level of transcription of the pIX gene relative to that of the major late gene was determined for each assay. Deletion of sequences located between −253 and −92 had no effect on the level of transcription (Fig. 1). Further deletion to −45 caused inactivation of transcription. Three ID mutant DNAs were not transcribed, as found in the in vivo assays. These results from in vitro studies were consistent with those obtained in vivo. Thus, the upstream element was required for the transcription of pIX DNA in vitro as well as in vivo, suggesting that the nuclear extracts from HeLa cells contained a factor(s) that recognized the upstream sequences located between −70 and −45 and activated transcription of the pIX gene. The requirement of the upstream element for transcription was observed reproducibly in reactions directed with both whole-cell and S100 extracts.

Presence of a specific repressor. To examine the presence of a factor specific for the upstream sequences of the pIX gene, proteins in nuclear extracts were fractionated by phosphocellulose column chromatography. Reconstitution of pIX gene transcription was carried out by using various combinations of the column fractions. A high level of transcription of pIX DNA was observed in the reaction containing all column fractions (Fig. 2, lane b). Almost the same level of transcription was obtained in the reactions lacking either the flowthrough or the 0.35 M KCl fraction (lanes c and d). No transcription of pIX DNA was observed in the reaction lacking either the 0.6 or 1.0 M KCl fraction (lanes e and f). This result might be attributable to the loss of a general transcription factor, since the major late gene was not transcribed either. Although a slight reduction in transcription of pIX DNA was seen in the reaction lacking both the flowthrough and the 0.35 M KCl fractions, this result was not specific for transcription of pIX DNA, as seen by the reduction in transcription of the major late DNA (lane g). Since these results did not rule out the possibility that factors specific for pIX DNA might have been recovered in the 0.6 or 1.0 M KCl fraction, similar assays were carried out for pIX DNA deleted of the upstream element. In these experiments, the level of transcription of the mutant pIX DNA was expected to be much lower than that of the wild-type DNA even in the reaction containing all column fractions. Surprisingly, however, almost identical levels of pIX DNA transcription were reconstituted for the mutant DNA relative to that for the wild-type pIX DNA by any combination of the column fractions (Fig. 2, lanes h to n). These results

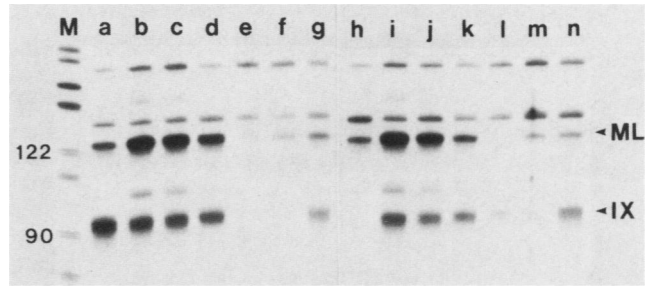

FIG. 2. In vitro transcription by column fractions. HeLa nuclear extracts were applied to a phosphocellulose column, and proteins were eluted as described in the text. RNA synthesized in vitro was analyzed by single-strand nuclease mapping. Lanes: a and h, nuclear extracts; b and i, all column fractions; c and j, minus the 0.1 M KCl fraction; d and k, minus the 0.3 M KCl fraction; e and l, minus the 0.6 M KCl fraction; f and m, minus the 1.0 M KCl fraction; g and n, minus the 0.1 and 0.3 M KCl fractions. DNA from pSVIX (lanes a to g) or pSVIX5215 (lanes h to n) was used as the template. Transcription of the major late gene served as the internal standard.

appeared to demonstrate that activity specifically affecting transcription of pIX DNA was not present in any of the fractions. The observed transcription of both types of DNA was dependent only on the TATA box. It should be noted that the transcription level of the pIX gene relative to that of the major late gene was significantly lower in the reaction containing all of the column fractions than in that containing nuclear extracts (Fig. 2, compare lanes a and b).

To examine whether the nuclear extracts contained activity specifically affecting transcription of pIX DNA, the effects of adding nuclear extracts to the combined column fractions were analyzed. When two transcription systems were mixed, transcription was additive or stimulative with respect to the wild-type pIX DNA template (Fig. 3). When the mutant pIX DNA was used as template, however, transcription directed by the column fractions was almost completely inhibited by addition of nuclear extracts. These results suggested that the nuclear extracts contained a factor(s) that specifically repressed transcription initiation in the absence of the upstream sequences of pIX DNA.

Before identifying this inhibitory activity, it was essential to rule out a possible artifact. In the experiments described above, supercoiled circular DNA was used as the template. If the inactive DNase in nuclear extracts is activated during column chromatography and if the upstream element of the pIX gene is an entry site for RNA polymerase II, nicks

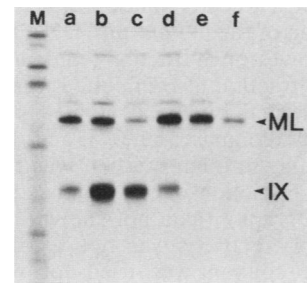

FIG. 3. Effects of mixing two in vitro transcription systems. In vitro transcription was carried out with 12.5 μl of nuclear extract (lanes c and f), 12.5 μl of the combined column fractions (lanes a and d), or both (lanes b and e). Lanes a to c, pSVIX DNA; lanes d to f, pSVIX5215 DNA.

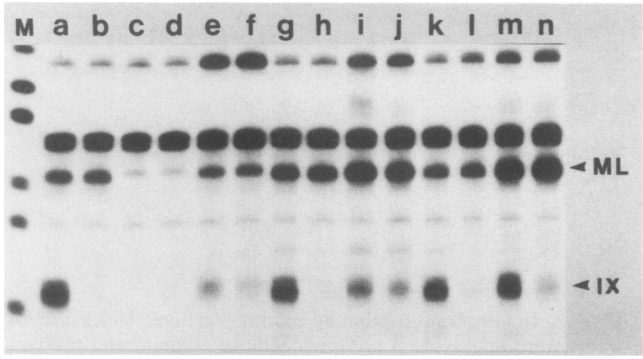

FIG. 4. Presence of a negative factor in nuclear extracts. Nuclear extracts were fractionated on a DEAE-cellulose column as described in Materials and Methods. Transcription was carried out with nuclear extracts (lanes a and b), with the flowthrough fraction (lanes c and d), with the 0.4 M KCl fraction (lanes e and f), with the flowthrough and 0.4 M KCl fractions (lanes g and h), with the combined P-11 fractions (lanes i and j), with the flowthrough fractions and the combined P-11 fractions (lanes k and l), and with the 0.4 M KCl fraction and the combined P-11 fractions (lanes m and n). DNA from pSVIX (lanes a, c, e, g, i, k, and m) or pSVIX5215 (lanes b, d, f, h, j, l, and n) was used as a template.

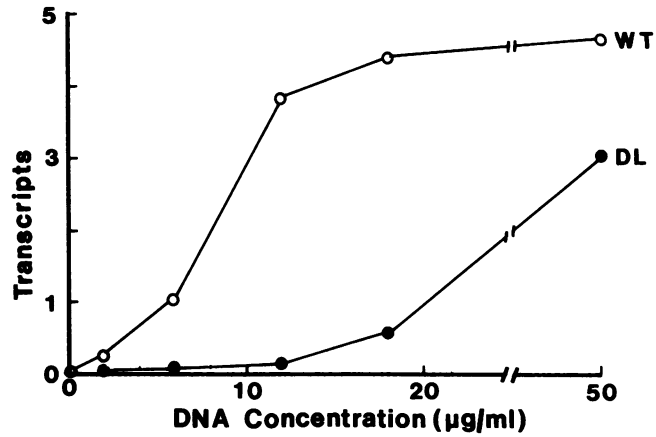

FIG. 5. Dose effects of template DNA or in vitro transcription. Transcription was performed with increasing amounts of pSVIX or pSVIX5215 DNA in a reaction containing 20 μl of nuclear extract. The amount of transcript was determined by densitometry of the autoradiograms and represented arbitrarily after normalization to the amount of the major late RNA.

introduced within the template DNA in the reconstituted system could provide artificial entry sites; in this case, transcription of pIX DNA could occur in the absence of the upstream element. This possibility was ruled out by the finding that inhibition of transcription of the mutant pIX DNA also occurred in nuclear extracts when linearized DNA was used as a template (data not shown). The results obtained in the mixing experiments shown in Fig. 3 also ruled out this possibility.

Although the presence of inhibitory activity was demonstrated in nuclear extracts, such activity was not detected in any fraction from the phosphocellulose column. It was essential to isolate the inhibitory activity in order to understand how this activity represses transcription specifically of pIX DNA deleted of the upstream element. When the nuclear extract was fractionated by DEAE-cellulose column chromatography, the 0.4 M KCl fraction by itself directed transcription of both the wild-type pIX DNA and the major late gene (Fig. 4, lanes c to f). Like the combined phosphocellulose column fractions, the 0.4 M KCl fraction directed transcription of both the mutant pIX and the wild-type DNA (lane f), the former somewhat less efficiently. When the flowthrough fraction, which did not direct transcription of pIX DNA, was added to the reaction containing the 0.4 M KCl fraction, stimulation of transcription of the wild-type pIX DNA was observed (compare lanes e and g). In contrast, transcription of the mutant pIX DNA was inhibited by addition of the flowthrough fraction (compare lanes f and h). The flowthrough fraction also inhibited transcription of the mutant DNA directed by the combined phosphocellulose column fractions (compare lanes j and l). The activity observed in the flowthrough fraction was recovered in the 0.6 to 1.0 M KCl fraction of the second phosphocellulose column chromatography (data not shown). It is not known why the inhibitory activity was not recovered when the phosphocellulose column was used for the first column chromatography. The inhibitory activity was also recovered in the single fraction after centrifugation through a 30 to 50% glycerol density gradient (data not shown). These results indicated that nuclear extracts from HeLa cells contained an activity inhibiting specifically the TATA box-dependent transcription of pIX DNA. This, in turn, suggested that the upstream element of pIX DNA functions to suppress this repression and consequently activates transcription.

Addition of nuclear extracts to the reaction containing the column fractions caused stimulation of transcription of wild-type pIX DNA (Fig. 3). Moreover, the flowthrough fraction of the DEAE-cellulose column also stimulated transcription directed by the 0.4 M KCl DEAE fraction or the phosphocellulose column fractions (Fig. 4, compare lanes e and g and lanes i and k). Comparison of the relative levels of transcription of pIX DNA and the major late DNA indicated that the HeLa cell extract contained another activity that stimulated transcription, depending on the presence of the upstream element of pIX DNA. It is also possible, however, that the stimulation resulted from complementation of limiting amounts of a basic transcription factor(s).

Possible mechanism for control of transcription. Since transcription was repressed by an inhibitory factor(s) only when the upstream element was removed, interaction of the factor with DNA would be expected at some region downstream of −41. To examine this possibility, the effects of increasing amounts of template DNA on transcription were analyzed. As the amount of wild-type pIX DNA increased, the amount of pIX transcript increased, reaching a plateau at 12 μg of template DNA per ml in 10 μl of nuclear extract (Fig. 5). In contrast, transcription of the mutant pIX DNA was not observed at low concentration of template DNA at which transcription of the wild-type pIX DNA was easily observed. However, at a higher template DNA concentration, the transcription level increased and approached that of the wild-type pIX DNA. Obviously, more template DNA was required when more nuclear extract was used. Since the total DNA concentration in the reaction was adjusted with pBR322 DNA, the observed suppression of transcriptional repression resulted from the increase of the mutant pIX DNA itself. These results, therefore, strongly suggest that an inhibitory factor in the HeLa nuclear extracts interacted with a certain region downstream from −41 of pIX DNA.

To identify the site responsible for repressing transcription, effects of deletion of the 3′ downstream region were analyzed in vivo and in vitro. Preliminary studies showed that deletion of the downstream region did not significantly

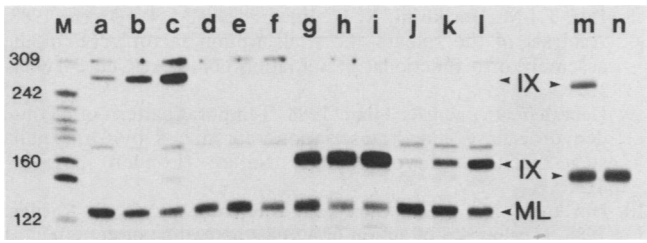

FIG. 6. Effects of deletion of the downstream region on transcription. In vitro transcription was carried out on DNA from pSVIX (lanes a to c), pSVIX5215 (lanes d to f), pSVIX3201 (lanes g to i), or pSVIX3201-1 (lanes j to l). The DNA concentration was 6 (lanes a, d, g, and j), 12 (lanes b, e, h, and k), or 18 (lanes c, f, i, and l) μg/ml. HeLa cells were transfected with pSVIX and pSVIX3201 (lane m) and pSVIX5215 and pSVIX3201-1 (lane n). After 48 h, RNA was prepared and analyzed as for Fig. 1. The hybridization probe used was a fragment covering −44 to +270. The transcripts from pSVIX and pSVIX5215 or pSVIX3201 and pSVIX3201-1 were expected to protect 270 or 158 nucleotides of the probe.

affect the level of transcription (Fig. 6, lane m, and data not shown). In these experiments, effects of the deletion on transcription of pIX DNA containing the upstream element were analyzed. Since the function of the upstream element might be to suppress transcriptional repression, pIX DNA lacking the upstream element would be suitable for analyzing the function of the downstream region. The level of transcription of pSVIX3102 DNA, deleted of the sequence between +33 and +122, was higher than that of the wild-type pSVIX DNA in vitro (Fig. 6, compare lanes a to c and lanes g to i). Stimulation of transcription by deletion of the downstream sequence was greater at low concentrations of template DNA. Furthermore, pSVIX3102-1 DNA, deleted of both the upstream and downstream sequences, was transcribed as efficiently as the wild-type DNA even under conditions in which pSVIX5215 DNA, deleted of the upstream element, was not transcribed (Fig. 6, compare lanes a to c and lanes j to l). When transcription of the same deletion pIX DNA was analyzed in vivo, little enhancement of transcription by deletion of the downstream sequence was observed in the presence of the upstream element (lane m). In contrast, in the absence of the upstream element, transcription of pIX DNA was significantly activated by the deletion (lane n). These results indicated that a sequence element within the region from +33 to +122 inhibited transcription of pIX DNA deleted of the upstream element. In light of these results, it was concluded that transcription of pIX DNA was controlled negatively by interaction between a repressive factor and the downstream element and that the upstream element was required for the suppression of transcriptional repression.

DISCUSSION

In this work, transcription of the pIX gene of adenovirus 2 was shown to be controlled by three *cis*-acting elements. Two of them, the TATA box and the upstream element, are positive elements, since their deletion resulted in an inactivation of transcription. The requirement of these two elements for transcription was also reproduced in an in vitro transcription system directed by a HeLa nuclear extract. As with other eucaryotic genes, the upstream element of pIX DNA was initially expected to activate transcription simply by interacting with a specific positive *trans*-acting factor. However, this was not the case. Fractionation of proteins in nuclear extracts by column chromatography revealed that nuclear extracts contained an activity inhibiting transcription specifically of pIX DNA lacking the upstream element. In addition, the TATA box of pIX DNA by itself could direct initiation of transcription in the reaction not containing such an inhibitor. Therefore, inactivation of transcription in vivo by deletion of the upstream element appears to be a manifestation of dormant repression of transcription. In other words, activation of transcription by the upstream element results from suppression of transcriptional repression. In this sense, the function of the upstream element of pIX DNA appears to be different from the functions reported for other eucaryotic genes.

Consistent with this view, another *cis*-acting element of pIX DNA was found to be a negative element responsible for transcriptional repression. Deletion of the downstream sequence between +33 and +122 resulted in stimulation of pIX DNA transcription in vitro irrespective of the presence of the upstream element. Analysis of transcription on other 3′-deletion mutants showed that such a negative element was localized within the region from +45 to +117 (data not shown), although the precise location has not been determined. The effect of deletion of the downstream element was more apparent in the absence of the upstream element both in vitro and in vivo. This result is in agreement with that obtained in in vitro reconstitution experiments, suggesting that the upstream element is a suppressor of transcriptional repression.

Negative regulatory elements identified in eucaryotic genes are often located adjacent to positive regulatory elements. Transcription of these genes is thought to be controlled by competitive binding of two *trans*-acting factors, positive and negative, to the elements (for a review, see references 1 and 15). In contrast to such genes, the pIX gene of adenovirus 2 was found to contain a negative element located in a region downstream of the cap site. Although it is not known whether this negative element functions in a position- and orientation-independent manner, a characteristic feature of the element is that it exerts its function when the upstream positive element is removed. Since transcription of pIX DNA containing the upstream element occurred irrespective of the presence of the downstream element, the upstream element appears to suppress transcriptional repression by the downstream element. Generally, upstream elements proximal to the TATA box are thought to facilitate binding of factor TFIID to the TATA box so as to activate transcription (8, 10, 11, 24). Preliminary experiments showed that (i) activation of pIX DNA transcription by the upstream element was inhibited by changing the distance from the TATA box, suggesting a direct interaction between these two elements, and (ii) a specific factor(s) bound to a DNA fragment containing the upstream element (unpublished observations). Given that no *cis*-acting element other than the TATA box was found within the region between −41 and +45, the results suggest that the downstream element may inhibit binding of TFIID to the TATA box by interacting with a negative factor (transcription repression) and that the upstream element may negate this repression by stimulating TFIID to bind stably to the TATA box (suppression of transcriptional repression). Alternatively, the downstream element, by binding with the negative factor, may inhibit elongation of transcription. Since the level of transcription of the wild-type pIX DNA was lower than that of a mutant pIX DNA lacking the downstream element, it is possible that transcription of pIX DNA is partially repressed even in the presence of the upstream element and that this

repression is due to inhibition of transcription elongation. In this case, it is unclear how the upstream element negates the effect of the downstream element. A direct interaction between the two elements could be postulated. To analyze in detail the mechanism of regulation of transcription initiation of pIX DNA, it is essential to purify the factors binding to each element.

Since the negative factor demonstrated in this study is present in HeLa cells, there may be some other cellular genes that are regulated by the same mechanism that regulates the pIX gene. Most of the eucaryotic genes studied so far appear not to have a negative element similar to that identified in the pIX gene. In most genes, the upstream element activates transcription by binding to the positive *trans*-acting factor. The TATA boxes of these genes by themselves appear not to direct transcription, and thus the upstream activating element is essential for transcription. In contrast, the TATA box of the pIX gene by itself could efficiently direct transcription in the absence of a negative factor. Therefore, genes with such a TATA box may need to acquire a negative regulatory system for inhibiting constitutive transcription by the TATA box, although the physiological significance of this requirement is not known. If, as discussed above, a direct interaction between the downstream element and the TATA box is responsible for transcriptional repression, there should be some heterogeneity in function among the TATA boxes. Simon et al. (26) recently demonstrated that multiple, functionally distinct TATA boxes can be distinguished by differences in inducibility of the adenovirus E1A gene. It is important, therefore, to determine whether the negative element identified within the pIX gene can influence the TATA boxes of other genes. In vitro-reconstituted transcription systems will be extremely powerful tools for examining the possibilities discussed above.

ACKNOWLEDGMENTS

I thank H. Saiga for helpful discussion, M. Muramatsu, H. Saiga, and N. Janjua for comments on the manuscript, and M. Oka for skillful technical assistance.

This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

LITERATURE CITED

1. **Atchison, M. L.** 1988. Enhancers: mechanisms of action and cell specificity. Annu. Rev. Cell Biol. **4**:127–153.
2. **Coffino, P. B., S. G. Knowles, S. G. Nathenson, and M. D. Scharff.** 1971. Suppression of immunoglobulin synthesis by cellular hybridization. Nature (London) **231**:87–90.
3. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
4. **Dynan, W. S., and R. Tjian.** 1985. Control of eukaryotic mRNA synthesis by sequence-specific DNA binding proteins. Nature (London) **316**:774–778.
5. **Garcia, J. A., F. K. Wu, R. Mitsuyasu, and R. B. Gaynor.** 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J. **6**:3761–3770.
6. **Goodburn, S., H. Burstein, and T. Maniatis.** 1986. The human β-interferon gene enhancer is under negative control. Cell **45**:601–610.
7. **Greenberg, A., R. Ber, Z. Kra-Oz, and R. Laskov.** 1987. Extinction of expression of immunoglobulin genes in myeloma × fibroblast somatic cell hybrids. Mol. Cell. Biol. **7**:936–939.
8. **Hai, T., M. Horikoshi, R. G. Roeder, and M. R. Green.** 1988. Analysis of the role of the transcription factor ATF in the assembly of a functional preinitiation complex. Cell **54**:1043–1051.
9. **Heberlein, U., and R. Tjian.** 1988. Temporal pattern of alcohol dehydrogenase gene transcription reproduced by Drosophila stage-specific embryo extracts. Nature (London) **331**:410–415.
10. **Horikoshi, M., M. F. Carey, H. Kakidani, and R. G. Roeder.** 1988. Mechanism of action of a yeast activator: direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. Cell **54**:665–669.
11. **Horikoshi, M., T. Hai, Y. S. Lin, M. R. Green, and R. G. Roeder.** 1988. Transcription factor ATF interacts with TATA factor to facilitate establishment of a preinitiation complex. Cell **54**:1033–1042.
12. **Killary, A. M., and R. E. K. Fournier.** 1984. A genetic analysis of extinction: transdominant loci regulate expression of liver specific traits in hepatoma hybrid cells. Cell **38**:523–534.
13. **Laimins, L., M. H. Konig, and G. Khoury.** 1986. Transcriptional "silencer" element in rat repetitive sequences associated with the rat insulin 1 gene locus. Proc. Natl. Acad. Sci. USA **83**:3151–3155.
14. **Larsen, P. R., J. W. Harney, and D. D. Moore.** 1986. Repression mediates cell-type-specific expression of the rat growth hormone gene. Proc. Natl. Acad. Sci. USA **83**:8283–8287.
15. **Linzer, D.** 1986. Negative transcriptional regulation of c-myc. Trends Genet. **2**:195–196.
16. **Maniatis, T., S. Goodbourn, and J. A. Fisher.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**:1237–1245.
17. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA **77**:3855–3859.
18. **Matsui, T.** 1982. In vitro accurate initiation of transcription on the adenovirus type 2 IVa2 gene which does not contain a TATA box. Nucleic Acids Res. **10**:7089–7101.
19. **Matsui, T., M. Murayama, and T. Mita.** 1986. Adenovirus 2 peptide IX gene is expressed only on replicated DNA molecules. Mol. Cell. Biol. **6**:4149–4154.
20. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46**:795–805.
21. **Nir, U., M. D. Walker, and W. J. Rutter.** 1986. Regulation of rat insulin 1 gene expression: evidence for negative regulation in nonpancreatic cells. Proc. Natl. Acad. Sci. USA **83**:3180–3184.
22. **Periman, P.** 1970. IgG synthesis in hybrid cells from an antibody-producing mouse myeloma and L-cell substrain. Nature (London) **228**:1086–1087.
23. **Remmers, E. F., J. Q. Yang, and K. B. Marcu.** 1986. A negative transcriptional control element located upstream of the murine c-myc gene. EMBO J. **5**:899–904.
24. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
25. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eukaryotic gene transcription. Trends Genet. **1**:224–230.
26. **Simon, M. C., T. M. Fische, B. L. Benecke, J. P. Nevins, and N. Heintz.** 1988. Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1A regulation. Cell **52**:723–729.
27. **Venkatesh, L. K., and G. Chinnadurai.** 1987. Activation of the adenovirus 2 protein IX by DNA replication in a transient expression assay. Nucleic Acids Res. **15**:2235–2250.
28. **Wasylyk, B., J. L. Imler, B. Chatton, C. Schatz, and C. Wasylyk.** 1988. Negative and positive factors determine the activity of the polyoma virus enhancer domain in undifferentiated and differentiated cell types. Proc. Natl. Acad. Sci. USA **85**:7952–7956.
29. **Weil, P. A., D. S. Luse, J. Segall, and R. G. Roeder.** 1979. Selective and accurate initiation of transcription at the major

late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell **18**:469–484.

30. **Weiss, M. C., and M. Chaplain.** 1971. Expression of differential functions in hepatoma cell hybrids: reappearance of tyrosine aminotransferase induction after the loss of chromosomes. Proc. Natl. Acad. Sci. USA **68**:3026–3030.
31. **Zinn, K., and T. Maniatis.** 1986. Detection of factors that interact with the human β-interferon regulatory region in vivo by DNAse I footprinting. Cell **45**:611–618.
32. **Zinn, K., D. DiMajio, and T. Maniatis.** 1983. Identification of two distinct regulatory proteins adjacent to the human β-interferon gene. Cell **34**:865–879.