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The Product of the Adenovirus Intermediate Gene IX Is a Transcriptional Activator

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We have investigated the functional properties of the product of the adenovirus type 5 gene IX. This gene, which is expressed at intermediate times postinfection, encodes a small polypeptide (pIX) of 140 residues that has previously been shown to be incorporated into the viral capsid. Here, we show that pIX, in addition to its structural contribution, exhibits transcriptional properties. In transient transfection experiments, expression of pIX stimulated adenovirus major late promoter activity. The effect was independent of other viral proteins, but the level of promoter activation appeared strongly pIX dose dependent; similar levels of induction were observed with other cellular or viral TATA-containing (but not with TATA-less) promoters. This promoter specificity could be reproduced in a cell-free transcription system by the addition of purified recombinant pIX, further stressing the transcriptional nature of the phenomenon. A preliminary structural analysis of pIX indicated that the integrity of a putative leucine zipper at the carboxy-terminal end of the molecule, as well as elements within the amino-terminal half, was critical for pIX transcriptional activity. The relevance of these findings in adenovirus infection is discussed.

Genes transcribed by RNA polymerase II (RPII) typically contain common core promoter elements that are recognized by a universal set of proteins, termed the general transcription factors (GTFs), and gene-specific DNA elements (enhancers and silencers) that are bound by cell-type-specific and gene-specific regulatory factors. The most common of the core promoter elements are the TATA box (consensus TATAa/tAa/t), which is located around position −30, and a pyrimidine-rich initiator (Inr; consensus YYANt/aYY), which is located at or near the transcription start site. TFIID is the only GTF capable of sequence-specific binding to eukaryotic promoter DNA, with one component (TBP) directly binding the TATA element (for a review, see reference 13) and certain TBP-associated factors directly (13) or indirectly (12) implicated in sequence-specific DNA binding.

The GTFs represent the ultimate targets of various gene-specific activators and repressors. The following intermediary factors that convey signals from regulatory factors to transcription proteins have been identified: (i) TAFs, which are components of TFIID (22, 57); (ii) general cofactors, which, in contrast to TAFs, do not associate tightly with GTFs but stimulate transcription in conjunction with a complete set of general factors (15, 49); and (iii) mediators, which associate with RPII to constitute the so-called holoenzyme (37, 38, 53).

Viruses constitute useful integrated model systems for the study of eukaryotic gene regulation and have largely contributed during the last decade to our knowledge of the field. The adenovirus major late promoter (MLP), a TATA- and Inr-containing promoter, has been most extensively studied as a model for eukaryotic transcription. Similarly, the adenovirus infection cycle constitutes an attractive system for the study of temporal control of transcriptional activity. Several early (E1a, E1b, E2, and E4) (for a review, see reference 2) and intermediary (IVa2) transcription units (58) encode proteins that have transcription regulatory properties. In the case of the IVa2 gene, its product (pIVa2) has recently been shown to be a component of proteins (DEF-A and DEF-B) that bind to sequence elements located downstream of the adenovirus MLP start site and contribute to MLP activation (43, 50). The adenovirus genome encodes another intermediate gene, gene IX, whose product (pIX) has been reported to be incorporated into the mature viral capsid, where it strengthens hexon nonamer interactions (10, 33, 59). Expression of pIX starts shortly after the onset of viral DNA replication, in contrast to the other adenovirus structural proteins which accumulate at much later times (23). This observation raises the possibility that before being assembled into the viral capsid, pIX may serve additional functions at the intermediate stage of the infection cycle.

With the example of pIVa2 in mind, we examined whether pIX may exhibit similar properties. We present evidence indicating that pIX is indeed a transcriptional activator and that it stimulates TATA-containing promoters of both viral and cellular origin.

MATERIALS AND METHODS

Cells and virus. HeLa cells were propagated as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 2.5% fetal calf serum and 2.5% newborn calf serum. Monolayer COS-7 cells were grown in 5% newborn calf serum-supplemented Dulbecco medium. HeLa or COS-7 cells (80% confluent) were infected with adenovirus type 5 (Ad5), at a multiplicity of infection of 20 PFU per cell in serum-free medium.

Recombinant protein IX production in bacteria. The nucleotide sequence encoding adenovirus protein IX was derived from the Ad2 genome by PCR amplification of a DNA fragment spanning coordinates 3600 to 4023. The amplified fragment was cloned into a derivative of pGEX-3X (Pharmacia), downstream of and in frame with the glutathione *S*-transferase (GST) sequence, generating the pGST-IX vector. Alternatively, this fragment was cloned into a derivative of pET-3a (55), downstream of and in frame with six histidine codons, generating the pH6-IX vector. The structures of both pGST-IX and pH6-IX vectors were verified by nucleotide sequencing.

Overnight starter cultures (15 ml) of *Escherichia coli* DH5α or BL21LysS, which were transformed with pGST-IX and pH6-IX, respectively, were inoculated into 500 ml of culture medium and grown at 37°C to an optical density at 600 nm of about 1. After isopropyl-thiogalactoside (IPTG) induction (0.5 mM; 2 h at 37°C), bacteria were collected. The pGST-IX-transformed bacteria were

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resuspended in phosphate-buffered saline (PBS) containing 2.5 ng each of leupeptin, pepstatin, aprotinin, antipain, and chymostatin (Sigma) per ml and disrupted by sonication. The resulting lysate was clarified by centrifugation (20 min at 10,000 × g, 4°C). The recombinant GST-IX fusion protein (rGST-IX) was purified from the cleared lysate by adsorption onto 1 ml of glutathione (GSH) agarose beads, successive washing with 10 ml of PBS and 10 ml of PBS containing 0.5 M NaCl, and elution with a buffer containing 50 mM Tris-HCl (pH 8.3), 5 mM GSH, and 20% glycerol. About 10 mg of rGST-IX protein was recovered.

The pH6-IX-transformed bacteria were resuspended in a buffer containing 10 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 0.1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM dithiothreitol, and 10% glycerol, and the suspensions were sonicated. The recombinant protein (rH6-IX), which was essentially produced as inclusion bodies, was solubilized in 6 M guanidinium-HCl–50 mM Tris-HCl (pH 7.5) and purified by chromatography over a 2-ml column of metal chelate affinity agarose (Qiagen, Pharmacia). The rH6-IX protein was eluted with 40 mM imidazole and renatured by successive dialysis in a buffer (20 mM Tris-HCl [pH 7.9], 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) containing 2, 1, 0.5, 0.25 M (1 h each) and no (14 h) guanidinium-HCl.

Eukaryotic expression vectors. The sequence encoding adenovirus protein IX (see above) or selected restriction fragments thereof were inserted into the pAT4 vector (gift from M. Vigneron), in a site located 3′ to the sequences encoding the F domain of the human estrogen receptor (hER) (4). In these vectors, the cytomegalovirus enhancer-herpes simplex virus type 1(HSV-1) thymidine kinase gene promoter of pAT4 directs the expression of a full-length (pAT4-IX) or deleted (pAT4-IXΔa and pAT4-IXΔb) protein IX that is tagged with the F epitope at its N terminus. The pAT4-IVa2 expression vector harboring the IVa2-coding sequence was constructed by inserting the IVa2 cDNA into the pAT4 plasmid. Various promoter sequences were subcloned in front of the chloramphenicol acetyltransferase (CAT) reporter gene of the promoterless pBLCAT6 vector (9) and were used as reporter plasmids in transfection experiments. Insertion of the Ad5 wild-type MLP sequence (−246 to +131, with respect to the MLP start site) or an MLP retaining only the TATA and Inr elements (−34 to +33) generated the MLP-CAT and MLPTATA-CAT reporters, respectively; insertion of Ad2 E2a (−59 to +40 [34]), Ad2 E4 (−148 to +30 [27]), the HSV-1 thymidine kinase (−105 to +51 [48, 61]), rabbit β-globin (−109 to +4 [60]), human ATFa (−212 to +83 [28]), mouse DP1 (−1012 to +167 [29]) and Ad5 E1a (−460 to +1) generated the pE2-CAT, pE4-CAT, pTK-CAT, pG-CAT, pATFa-CAT, pDP1-CAT, and pE1a-CAT reporters, respectively.

In vivo expression assay. COS-7 cells were transfected by calcium phosphate coprecipitation (17) of recombinant DNA vectors adjusted to 15 μg per 9-cm petri plate, with double-stranded carrier DNA (pBluescript). Thirty-six hours after transfection, the cells were harvested, extracts were prepared, and aliquots, normalized by protein concentration, were assayed for CAT activity as described elsewhere (30, 62). The percentages of chloramphenicol acetylation were determined from at least three independent experiments and were quantitated with a Bioimaging analyzer (Fuji Photo Film Co.).

In vitro runoff transcription. Transcription reactions were carried out under conditions previously described (50). The DNA templates were obtained by PCR amplification of the Ad5 MLP sequences between positions −137 and +180 (with respect to the MLP start site) or of the Ad5 E2a sequences between positions −128 and +119 (with respect to the E2a start site). Transcripts were analyzed by electrophoresis on 6% acrylamide-urea sequencing gels.

Antibodies, immunoblots, and fluorescence microscopy. Polyclonal antibodies were raised against CsCl-purified Ad2 virions (antivirion) and against the solubilized and purified rGST-IX protein (anti-pIX) by subcutaneous injection into rabbits. Monoclonal antibodies against the IVa2 protein (MAb1A1) have been previously described (43). Monoclonal antibodies against the F domain of the hER (MAb3A6) have been described elsewhere (4). Proteins that were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose filters were reacted with specific antibodies and revealed by a chemiluminescence detection system (DuPont NEN) as described elsewhere (8). Immunofluorescence staining experiments were carried out as previously described (44) with monolayer HeLa cells which were grown on glass coverslips and infected with Ad5 (20 PFU per cell).

RESULTS

Expression of pIX in Ad-infected cells. To examine the kinetics of pIX expression and its intracellular distribution during Ad infection, antibodies were raised against a recombinant pIX. To this end, pIX was overproduced in bacteria as a GST fusion protein, and the resulting chimeric protein (rGST-IX) was purified by GSH affinity chromatography. The GSH eluate (Fig. 1A, lane 4), which was highly enriched in the 40-kDa recombinant protein, was used to immunize rabbits.

The specificities of the anti-pIX antibodies were first tested with the rGST-IX protein (Fig. 1B). While no protein was detected in extracts from noninduced bacteria (lane 1), a strong band at about 40 kDa was revealed in extracts from induced bacteria and in the GSH eluate of the affinity chromatography (lanes 2 and 3, respectively). Additional, weaker signals were also observed at lower molecular masses, most likely corresponding to spontaneous cleavage products of the GST fusion protein in the bacterial extracts.

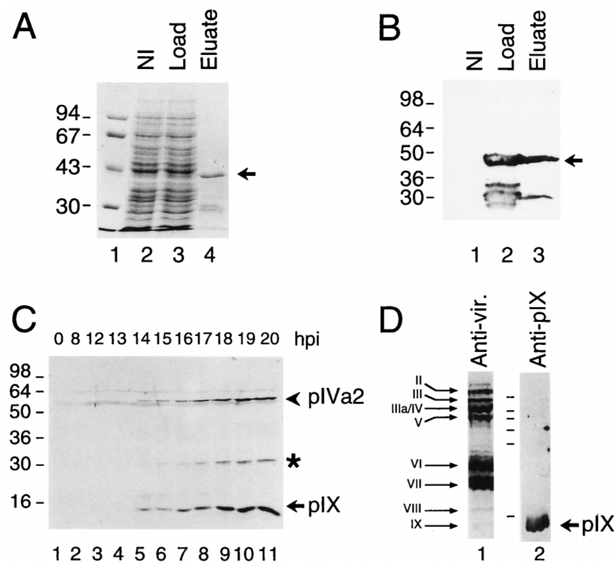

FIG. 1. Immunodetection of pIX. (A) The recombinant GST-IX fusion protein (rGST-IX) used to raise rabbit polyclonal (anti-pIX) antibodies was produced in *E. coli* after transformation with the pGST-IX vector and was purified by one-step affinity chromatography over GSH agarose. A crude extract from noninduced bacteria (NI) or a soluble protein extract from IPTG-induced bacteria (load) (2-ml equivalents of initial culture) was analyzed next to the GSH eluate (eluate) by SDS-10% PAGE and stained with Coomassie blue. Molecular mass standards (Pharmacia) were run in parallel (lane 1), with sizes (in kilodaltons) indicated on the left. Arrow, the full-length rGST-IX fusion protein. (B) Proteins from a gel similar to that shown in panel A blotted onto nitrocellulose and probed with anti-pIX antibodies. Prestained SeeBlue size markers (Sovex) are positioned on the left (in kilodaltons). Arrow, the full-length rGST-IX fusion protein. (C) HeLa cells that were mock infected (lane 1) or that were infected with Ad5 (multiplicity of infection of 20 PFU per cell [lanes 2 to 11]). Infected cells were collected at 8 h p.i. and at 1-h intervals from 12 to 20 h p.i. Extracts were prepared and fractionated by SDS-PAGE, and specific proteins were revealed by immunoblot analysis by successively using the anti-pIX polyclonal antibodies (indicated by the asterisk and the arrow) and the monoclonal antibody MAb1A1 against pIVa2 (indicated by the arrowhead). A minor band, which is also present in uninfected cells (lane 1) and which migrates slightly faster than pIVa2, corresponds to a nonspecific background signal. Size markers (in kilodaltons) are positioned on the left. (D) Ad5 virion particles, purified by CsCl density gradient centrifugation, were disrupted by boiling in SDS sample buffer, fractionated by SDS-12% PAGE, and blotted onto nitrocellulose. Separate parts of the blot were probed with anti-virion (lane 1) or with anti-pIX (lane 2) antibodies. Arrows on the left point to major virion polypeptides as revealed by the antibody. Positions of size markers (the same as in panel C) are indicated between lanes 1 and 2. Arrow on the right, position of pIX.

The time course of pIX expression during Ad infection was examined by immunoblot analysis of extracts prepared at different times postinfection (p.i.) (Fig. 1C). No bands were revealed in uninfected cell extracts or early in infection, further stressing the selectivity of the antibodies. As expected, pIX became detectable at the same time as pIVa2, another intermediary gene product; both proteins slowly accumulated after the onset of viral DNA replication, which, as determined in parallel with aliquots of the same cell extracts, occurred at about 12 h p.i. (not shown; see reference 43). An additional protein of about 30 kDa was detected with the anti-pIX antibody, but not with the anti-pIVa2 antibody alone (see reference 43); the nature of this protein, which appeared to be expressed with the same kinetics as those for pIX, is presently

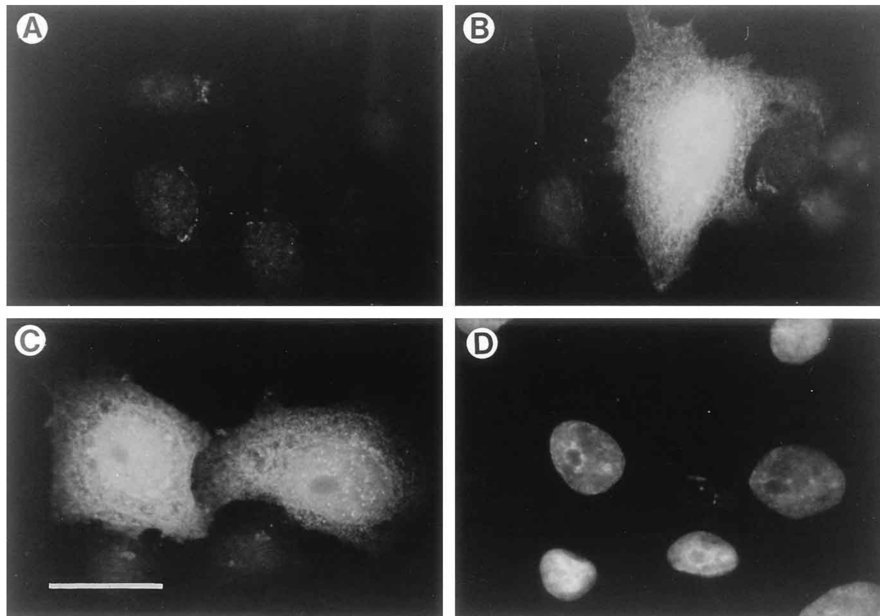

FIG. 2. Subcellular localization of pIX in Ad5-infected cells. HeLa cells were mock (A)- or Ad5 (B to D)-infected and fixed at 14 (B) and 17 (C and D) h p.i. After permeabilization, the cells were treated with anti-pIX polyclonal antibodies (immune serum diluted 1:5,000) and stained with Texas red-conjugated donkey anti-rabbit immunoglobulin G (diluted 1:500). (D) The cells shown in panel C but counterstained with Hoechst 33258, revealing additional, presumably uninfected cells within the field. Bar, 10 μm.

unknown but may correspond to dimers of pIX resistant to the SDS-PAGE denaturing conditions. In agreement with the earlier finding that pIX is a component of the viral capsid (10, 59), the anti-pIX antibody also revealed pIX in a preparation of mature virions that were purified by two successive CsCl gradient centrifugations (Fig. 1D).

Intracellular localization of pIX as revealed by immunofluorescence. Immunofluorescence staining of Ad-infected cells with pIX-specific antibodies revealed pIX expression from 14 h p.i. onwards (Fig. 2B and C), while no significant labelling was observed at earlier times p.i. or in mock-infected cells (Fig. 2A and data not shown). Although some staining was present in the cytoplasm, it was predominantly associated with the nuclei in positive cells, with a speckled distribution appearing at later times (17 h p.i. [Fig. 2C]; 20 h p.i. [not shown]). No obvious nuclear localization signal, whether comprised of a single block of basic amino acid residues or of two separate blocks (11, 21), could be identified within the pIX peptide sequence (see Fig. 7). Together, these observations suggest that due to its small size, pIX may freely diffuse from the cytoplasm to the nucleus but is likely to be retained within a specific nuclear structure.

Recombinant pIX stimulates MLP activity in transfected cells. The expression and accumulation of pIX, before full activation of the virus late transcription unit, raise the possibility that pIX serves functions in addition to participating in the viral capsid assembly. With the precedent of pIVa2 studies in mind, which revealed the contribution of this intermediate gene product in MLP activation (43, 50), we examined the effect of pIX expression on MLP activity. To this end, a pIX expression vector (pAT4-IX) was cotransfected into COS cells, together with a MLP-CAT reporter plasmid in which the CAT gene was driven by the wild-type MLP sequence. As shown in Fig. 3A, CAT expression is stimulated in a dose-dependent manner by pAT4-IX transfection (columns 1 to 5). The amount of pAT4-IX DNA required for maximal activation was remarkably low (100 ng per dish). Transfection of a plasmid based on the same vector sequences but expressing the viral pIVa2 protein, together with the MLP-CAT construct, had no effect on this reporter activity (compare columns 1 and 6), in agreement with earlier observations (58). Together with the fact that the overall amounts of pAT4 vector sequences were kept constant (see legend to Fig. 3), this rules out the remote possibility that vector sequences were responsible for the stimulation observed with pAT4-IX.

The pIX-induced stimulation of the MLP was only moderately affected by deletion of both upstream and downstream elements of the MLP (TATA), as shown in Fig. 3B. Since the reporter plasmid used in this experiment comprised a minimal MLP, extending from positions −34 to +33 (relative to the MLP start site), this result leads to the conclusion that the stimulatory effect of pIX observed here may be essentially mediated by the core promoter elements (TATA box and Inr elements) retained in this construction.

To examine whether the extent of MLP stimulation by pIX could be further enhanced by the presence of other virus or virus-induced cellular products, cells were transfected with the MLP-CAT reporter alone or together with the pAT4-IX expression vector and were overinfected with Ad5. The cells were harvested at different times p.i. (but before accumulation of virally expressed pIX), and the extracts were assayed for CAT activity (Fig. 3C). As expected, the level of expression from the MLP-CAT reporter increased with time p.i. (open columns), most likely as a result of accumulation of MLP transactivators such as E1a (40) and DEF (5, 7, 35, 41, 43, 45) during the course of infection. Upon cotransfection of the pIX expression vector (shaded columns), the level of reporter CAT activity was further increased. Interestingly, the extent of MLP stimulation, as reflected by the ratios of CAT activities measured in the presence and absence of the pIX vector, did not change significantly during the same period. This result indicates that pIX did not require additional infection-related compounds to exert its effect, at least during the time period p.i. tested.

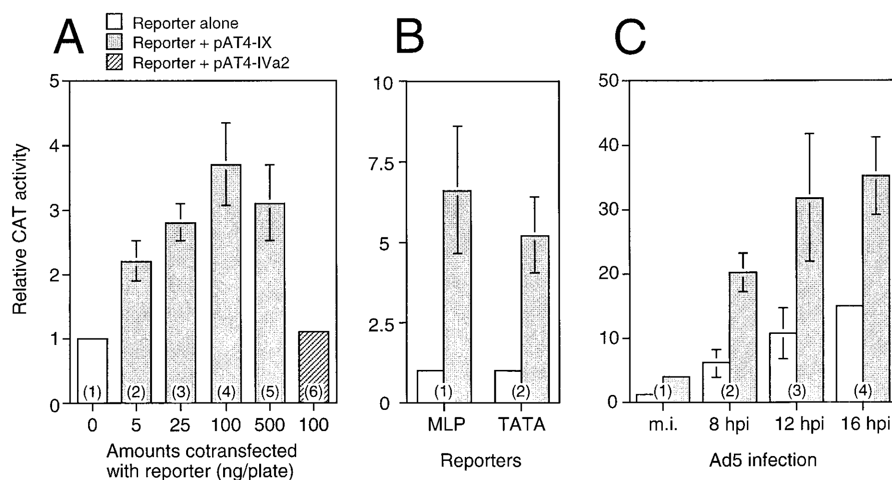

FIG. 3. MLP expression is activated by pIX. (A) The wild-type MLP-driven CAT reporter plasmid (MLP-CAT; 5 μg) transfected into COS-7 cells, either alone (open column 1) or with vectors expressing pIX (pAT4-IX; 5 to 500 ng [shaded columns 2 to 5]) or pIVa2 (pAT4-IVa2; 100 ng [striped column 6]). In each transfection, the final amount of pAT4 vector sequences was adjusted to 500 ng by the addition of empty pAT4. Relative CAT reporter activities (means from three independent experiments) are presented, with corresponding standard deviations. (B) COS-7 cells transfected with the pAT4-IX or pAT4-IVa2 vectors (100 ng), together with 5 μg of MLP-CAT reporters harboring either a wild-type MLP (MLP [columns 1]), an MLP with an altered upstream element (MLPmUE [columns 2]), or an MLP lacking both DE1 and DE2 downstream elements (MLPΔDE [columns 3]). Relative stimulations of CAT activities (in the presence of pAT4-IX) are presented as described above, after adjustment of the basal CAT values to one. (C) COS-7 cells transfected with the wild-type MLP-CAT reporter (5 μg), in the absence or presence (100 ng) of the pAT4-IX expression vector. Eighteen hours later, the same cells were infected with Ad5 (columns 2 to 4) or were mock infected (m.i. [columns 1]). The cells were collected at 8, 12, and 16 h p.i., and extracts were prepared. Relative CAT activities are presented as described above.

Stimulation of MLP activity by pIX is transcriptional. Stimulation of CAT activity, as measured from an appropriate transfected CAT reporter, is usually considered to reflect an increase in the level of corresponding RNA transcripts. To confirm that the pIX-mediated activation observed here corresponded to the accumulation of transcripts initiated at the MLP start site and not to a posttranscriptional effect, a quantitative primer extension assay based on oligonucleotide-primed reverse transcription of specific RNA molecules was used. Since the CAT transcripts appeared to be very short-lived and hard to detect, we performed the assay with cells that were transfected with an MLP-globin construct (Fig. 4A) whose transcripts were apparently more stable than those from the MLP-CAT reporter. As seen in Fig. 4B, transcripts of the expected length (102 nucleotides [nt]), initiating at MLP position +1, were detected. When the cells were cotransfected with the pAT4-IX vector, the relative intensity of the band corresponding to this extended primer increased to levels similar to those reached by the relative CAT activities (compare with Fig. 3B). This result further supports the conclusion that pIX is a transcriptional activator of the MLP. In addition, since in the reporter template used here the globin sequences were driven by a minimal MLP promoter, this result confirms that pIX mediates its effect through core promoter elements.

Differential responsiveness of promoters to pIX transactivation. The fact that a minimal MLP promoter retaining only the TATA box and initiator elements was still responsive to pIX (Fig. 3B and 4B) prompted us to test the effect of pIX in similar transfection assays with a series of other viral and cellular promoters (Fig. 5A). As shown in Fig. 5B, promoters like the ones from the Ad5 E4, the rabbit β-globin, and the HSV-1 thymidine kinase genes were clearly stimulated by pIX to levels similar to or higher than those for the MLP.

By contrast, pIX had no apparent effect on other promoters, such as those of the Ad5 E2, the human ATFa, and the mouse DP1 genes. The inhibition observed in the case of the ATFa and DP1 promoters was not specific, since a similar reduction in CAT activity was observed upon expression of the unrelated pIVa2 protein. It is possible that these promoters were particularly sensitive to potential competition effects of vector sequences or to squelching effects exerted by the overexpressed pIX or pIVa2 protein.

Strikingly, the series of pIX-responsive promoters all have a well-defined TATA box around position −30 (TATAAAA, TATATATA, CATAAAA, and TATTAAG for the MLP, E4, globin, and thymidine kinase promoters, respectively). By contrast, those which were unaffected by pIX are of the housekeeping type, with no identifiable TATA sequence. Whether

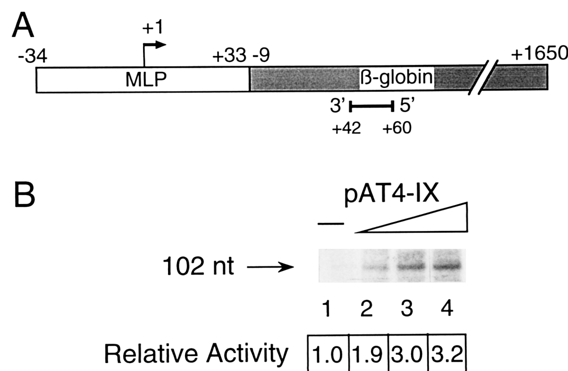

FIG. 4. Activation of MLP by pIX is transcriptional. (A) Schematic representation of the chimeric MLP-globin reporter which was used to generate stable transcripts. In this vector, the rabbit β-globin gene (positions −9 to +1650 with respect to the β-globin transcription start site) was linked to the wild-type MLP (positions −34 to +33 with respect to the MLP start site). The position of the primer (positions +42 to +60 with respect to the β-globin start site) used in the reverse transcriptase assay is indicated. (B) COS-7 cells transfected with this MLP-globin reporter (5 μg), either alone (lane 1) or with increasing amounts of pAT4-IX (10, 50, and 200 ng [lanes 2 to 4]). Total cytoplasmic RNA was isolated 36 h after transfection and used as a template in a reverse transcriptase primer extension assay, together with the globin-specific primer. The reaction generated a 102-nt DNA product that was visualized by autoradiography and quantitated by bioimaging. Relative intensities of the 102-nt band are given below each lane.

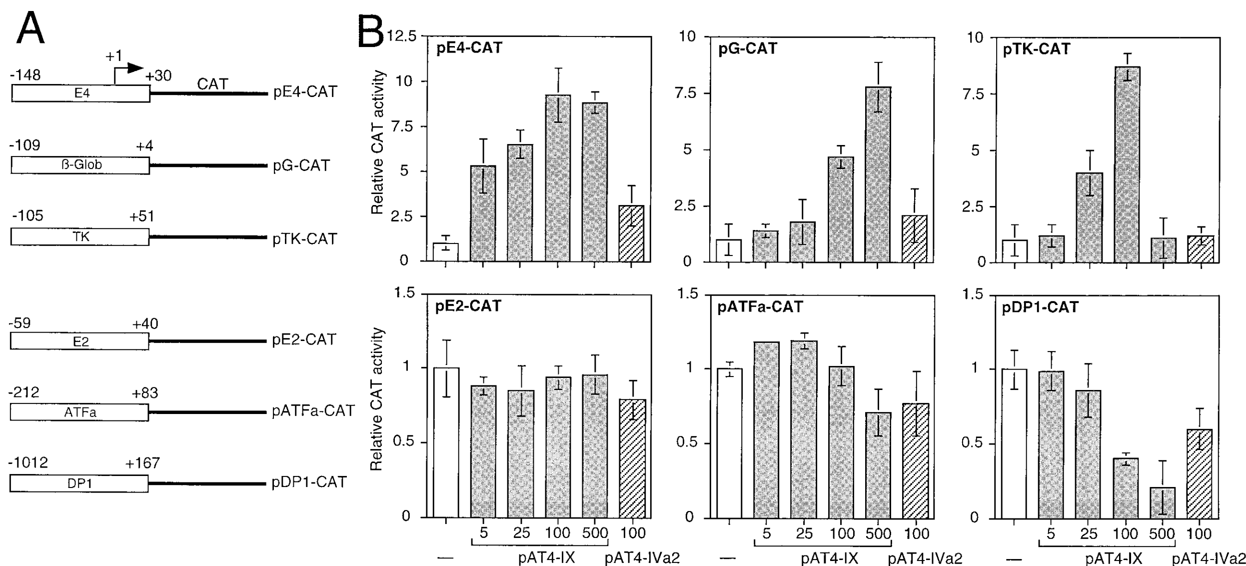

FIG. 5. Promoter specificity of pIX-mediated transactivation. (A) The structures of the chimeric CAT reporter plasmids used in this study are schematically depicted. Promoter regions (coordinates are given with respect to the corresponding start sites) of the Ad5 E2a (pE2-CAT) and E4 (pE4-CAT) transcription units, the rabbit β-globin (glob) gene (pG-CAT), the HSV-1 thymidine kinase (TK) gene (pTK-CAT), the human ATFa gene (pATFa-CAT), and the mouse DP1 gene (pDP1-CAT) were fused to the CAT gene. (B) COS-7 cells were transfected with these CAT reporter vectors (5 μg of each vector, except for pDP1-CAT, which was at 0.5 μg per plate), as indicated at the top of each panel, either alone (open columns) or together with pAT4-IX (5, 25, 100, and 500 ng [shaded columns]) or pAT4-IVa2 (100 ng [striped columns]). Relative CAT activities are presented as described in the legend to Fig. 3.

this distinction is responsible for the differential effect of pIX remains to be established.

In vitro runoff assays confirm the differential effect of pIX on promoter activity. We also tested the effect of pIX in cell-free transcription reactions, using whole-cell extracts as a source of RPII and general transcription factors. To keep the size of the recombinant pIX as close as possible to that of the natural protein, we decided to tag pIX with six histidine codons instead of GST. Histidine-tagged recombinant pIX (rH6-IX), which was produced in bacteria, was purified to homogeneity by affinity chromatography (Fig. 6A and B) and added to the reaction mixtures. A typical transcription assay performed in the presence of Ad5 MLP or E2a templates and with increasing quantities of rH6-IX is shown in Fig. 6C. The amounts of MLP runoff products (transcripts of around 180 nt) were clearly increased in the presence of rH6-IX, whereas this protein had no significant effect on the activity of the E2a promoter (bands at about 119 nt). These results not only emphasize the conclusion that pIX is a transcriptional activator but also further stress the promoter specificity of this effect.

The N-terminal region and the leucine zipper of pIX are essential for its activity. Examination of the pIX peptide sequence (Fig. 7A) revealed, at the C-terminal end of the molecule, a putative leucine-zipper comprising four leucine and one valine residues, each spaced seven residues apart. As a preliminary approach to delineate the functional domains of pIX, we manipulated the pAT4-IX expression vector (making use of unique restriction sites) to delete parts of the protein

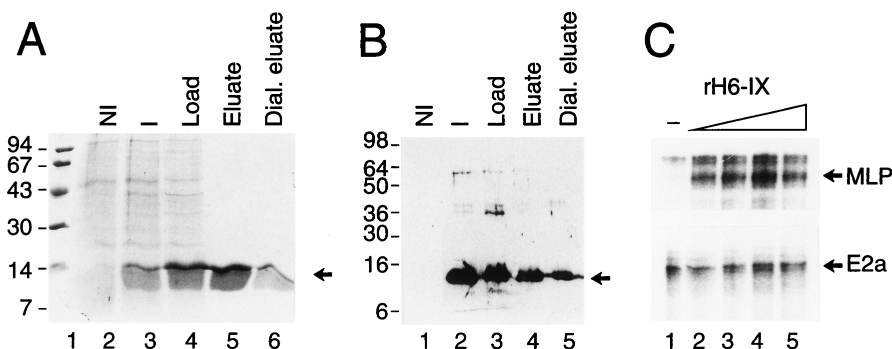

FIG. 6. Bacterially produced recombinant pIX transactivates the MLP in vitro. (A) Purification of the His-tagged recombinant protein IX by metal chelate affinity chromatography. Total lysates from 0.1-ml equivalents of bacterial cultures transformed with pH6-IX, prepared before (noninduced [NI]; lane 2) or after (induced [I]; lane 3) IPTG induction, were analyzed by SDS-12% PAGE, together with 5 μg of the guanidinium-chloride-solubilized inclusion bodies (load; lane 4) and 3 μg of the imidazole eluate, before (lane 5) and after (lane 6) protein renaturation. Protein size markers (in kilodaltons) were run in parallel (lane 1), and the gel was stained with Coomassie blue. Arrow, the His-tagged recombinant protein IX (rH6-IX). (B) Immunoblot analysis of metal chelate affinity chromatography. Proteins from a gel similar to that shown in panel A were blotted onto nitrocellulose and probed with anti-pIX antibodies. The positions of prestained protein size markers (in kilodaltons) are indicated on the left. (C) Cell-free transcription assays performed with a HeLa whole-cell extract, with Ad5 MLP (top) and E2a (bottom) sequences as templates. The reactions were carried out in the absence (lanes 1) or presence of increasing amounts of purified and renatured rH6-IX as indicated (5, 25, 100, and 500 ng per assay [lanes 2 to 5, respectively]). Specific runoff transcripts are indicated.

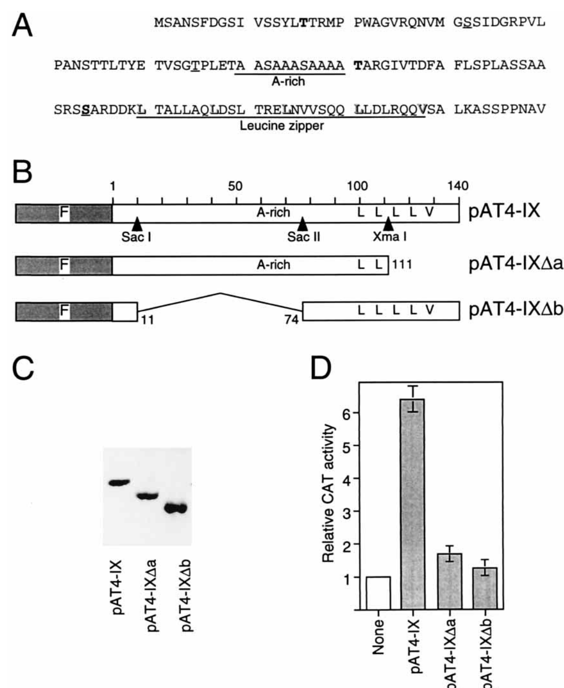

FIG. 7. Deletion analysis of pIX transcriptional activity. (A) Primary structure of pIX (140 amino acids), as derived from the Ad2 DNA nucleotide sequence (3), showing the putative leucine zipper region, an alanine-rich domain (A-rich), and potential target sites for protein kinase C (boldface letters) or casein kinase II (underlined letters). (B) Schematic representation of the structures of the pIX recombinants. Sequences encoding the full-length pIX (pAT4-IX) or two deleted versions thereof (pAT4-IXΔa and pAT4-IXΔb) were fused to the sequence of the F domain of the estrogen receptor into the pAT4 expression vector. The approximate positions of restriction sites used for deletion constructions, as well as of the A-rich and putative leucine zipper domains, are indicated. (C) COS-7 cells transfected with the pG-CAT reporter plasmid (5 μg) either alone (lane 1) or with each of the pIX recombinant vectors (amounts transfected per plate were adjusted to yield equivalent levels of protein expression [0.3, 2.2, and 0.2 μg of pAT4-IX, pAT4-IXΔa, and pAT4-IXΔb, respectively]). Cells were collected 36 h later, and extracts were prepared. Aliquots corresponding to 10 μg of cell lysates were fractionated by SDS-15% PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody (MAb3A6) directed against the F domain of the chimeric F-pIX proteins. Protein bands of the expected sizes were revealed. (D) CAT activities of the extracts were assayed, and relative levels of conversion of [^{14}C]chloramphenicol into its acetylated forms were determined.

(Fig. 7B). pAT4-IXΔa expressed a truncated protein lacking half of the leucine zipper; pAT4-IXΔb expressed a protein from which most of its N-terminal half was deleted and lacking an alanine-rich domain as well as the majority of potential phosphorylation sites.

When these vectors were transfected into COS cells, proteins of the expected sizes were produced, as revealed by immunodetection with antibodies directed against the common F domain fused to each recombinant protein (Fig. 7C). The transcriptional activities of these proteins were tested on the rabbit β-globin promoter by cotransfection of the globin-CAT reporter plasmid (pG-CAT). As shown in Fig. 7D, a deletion of the 28 C-terminal pIX residues, which removed part of the leucine zipper, severely impaired transactivation of pG-CAT. Similarly, deletion of the N-terminal half of pIX drastically reduced reporter stimulation. It appears, therefore, that both N- and C-terminal elements of pIX are critically involved in its transactivation function. Precise delineation of these elements will be informative.

DISCUSSION

In the present study, we report the transcriptional properties of pIX, the product of the adenovirus intermediate gene IX. This protein, which is ultimately incorporated into the viral capsid, stimulates the activity of the viral MLP. Interestingly, pIX is the only adenovirus structural protein that is not encoded by the major late transcription unit, which is a situation well in accordance with pIX contributing to the activation pathway of late gene transcription.

The transcriptional activity of pIX is independent of other adenovirus proteins. We have previously shown that pIVa2, another adenovirus intermediate gene product, was a component of proteins (DEF-A and DEF-B) that bind to sequence elements located downstream of the adenovirus MLP start site and contribute to MLP activation (43, 58). While DEF-B is composed of a homodimer of pIVa2, DEF-A is a heteromeric protein comprising pIVa2 and an additional component(s) that is not yet known. Since both intermediate genes (IVa2 and IX) exhibit identical expression kinetics during the course of viral infection, the possibility arose that pIX could be the partner of pIVa2 in DEF-A. We used the specific anti-pIX antibodies in band shift and immunoblot experiments with crude or partially purified DEF-A fractions to explore this possibility (not shown). However, all attempts to detect pIX within this complex failed, ruling out its direct involvement in downstream element-mediated MLP activation. This conclusion is further supported by transfection experiments which clearly indicate that the stimulatory effect of pIX was not enhanced by pIVa2 coexpression (42a). In fact, it was independent from any other viral or virus-induced protein, since the level of pIX-mediated stimulation was the same whether pIX was expressed in the context of noninfected or of Ad-infected cells.

The pIX protein interacts with a nuclear component(s). Virus transcription regulators can be tentatively classified according to their macromolecular interactions as follows: (i) viral proteins that recognize specific DNA target sequences (e.g., Ad pIVa2 [58] and papillomavirus E2 protein [6, 24, 47]); (ii) viral proteins that interact with cellular DNA-binding factors, thereby hooking to them an additional potent activation domain (e.g., Ad E1A [16, 32, 39, 42], HSV VP16 [25], and human T-cell leukemia virus tax1 [1, 46]); (iii) and viral proteins acting as cellular cofactors (e.g., HSV ICP4 [19], hepatitis B virus pX [31, 52], simian virus 40 large T [20]).

The mechanism of promoter activation by pIX is presently unknown. Our observation that only promoters with consensus TATA box elements, whether of viral or cellular origin, were responsive to pIX is striking; sequence comparison of these promoters did not reveal any other conserved promoter element. No specific DNA-binding activity of pIX has been detected so far, in agreement with the absence of any known structural motif involved in DNA binding. It is likely, therefore, that pIX operates by interacting with a promoter-bound factor(s) specifically recruited on TATA-containing promoters. In this respect, pIX may belong to either one of the last two classes of viral activators, as defined above.

Identification of the target protein(s) of pIX will help in understanding the mechanism of pIX-mediated transcriptional stimulation. The putative leucine zipper motif present within the C-terminal half of pIX may be part of the domain which contacts this component(s) of the transcription machinery, since its deletion severely impairs promoter activation. The fact that this deletion does not significantly affect the nuclear localization of pIX suggests that another domain, located within the N-terminal part of pIX, is predominantly involved in its nuclear retention (42a). It is not known at present whether

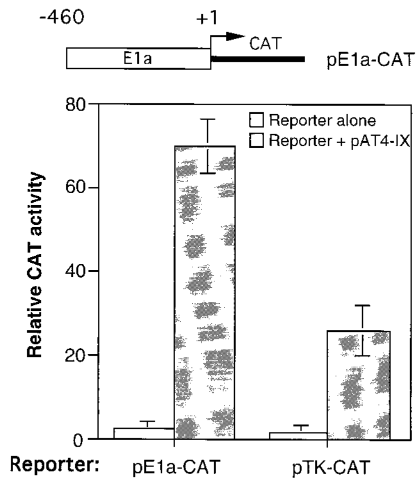

FIG. 8. Efficient stimulation of the viral E1a promoter by pIX. The structure of the chimeric pE1a-CAT reporter plasmid is schematically depicted; the promoter region of the Ad5 E1a transcription unit (between positions −460 and +1) was synthesized by PCR-mediated amplification, with appropriate oligonucleotide primers bearing *Hin*dIII and *Xho*I extensions, and inserted between the corresponding restriction sites of the promoterless pBLCAT6 vector (9). The resulting recombinant was isolated (pE1a-CAT), and its structure was verified by nucleotide sequencing. (Bottom graph) COS-7 cells were transfected with 5 μg of the pE1a-CAT and pTK-CAT (see Fig. 5) reporter vectors, either alone (open columns) or together with pAT4-IX (100 ng [shaded columns]). Relative CAT activities are presented as described in the legend to Fig. 3.

the potential phosphorylation sites and/or the alanine-rich element, both of which are situated within this half of the molecule, is responsible only for the nuclear retention of pIX or whether these elements also contribute to transcriptional activity.

Physiological implications of pIX transcriptional activity. The adenovirus deletion mutant *dl*313 (36) is defective for the products of the E1 region and *gene* IX; this virus has a significantly reduced packaging limit and a thermolabile virion phenotype but otherwise propagates with nearly wild-type efficiency in 293 cells, a cell line which provides the virus E1 functions, but not pIX (14, 18, 26, 56). This raises the question concerning the physiological significance of the transcriptional activity of pIX. pIX may contribute to efficient induction of the late transcription unit, as suggested by the present study. Alternatively, or in addition, the capsid pIX molecules that are internalized during endocytosis of virus particles upon cell infection may directly participate in induction of the viral transcription program by activating the promoter of the E1a immediate-early gene. The observation (Fig. 8) that pIX most efficiently stimulated the activity of the TATA-containing E1a promoter, when assayed under our transfection conditions, supports this line of reasoning. There are other examples of transactivators that are carried along with the virion particle, the herpesvirus VP16 representing the best-characterized precedent (see reference 51 and references therein). However, pIX is particular in that it is a component of the virion located on the outer surface of the capsid, as revealed by cryoelectron and difference imaging (54).

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P.L. and M.R..-C. should be considered as equal first authors.

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