Adenovirus E1a Gene Product Expressed at High Levels in *Escherichia coli* Is Functional

Abstract. *The human type C adenovirus E1a 13S messenger RNA encodes a gene product, that positively regulates the transcription of viral genes and certain cellular genes and is involved in the transformation of primary mammalian cells. The E1a gene product was expressed at high levels in* Escherichia coli. *In a* Xenopus *oocyte microinjection assay, the purified* Escherichia coli*–produced protein activated the E1a-responsive adenovirus E3 promoter and functioned as efficiently as the E1a gene itself.*

The human adenovirus E1a region encodes a product that enhances the expression of the early viral transcription units (*1–4*). E1a also activates the expression of at least one host cell gene (*5*). Moreover, the E1a gene is sufficient for the immortalization of primary cells and, in combination with certain other oncogenes, can transform primary cells (*6, 7*). The E1a transcription unit encodes two overlapping messenger RNA's (mRNA's), 12*S* and 13*S*, which share identical 5′ and 3′ termini and differ internally as a result of differential splicing (*8, 9*). Studies with various E1a mutants show that the product of the 13*S* mRNA alone is required for transcriptional activation of early viral genes (*1, 10*).

Detailed analysis of the E1a gene product has been difficult since this protein is produced at relatively low levels in adenovirus-infected or -transformed cells. To obtain sufficient quantities of E1a for both biochemical and physical analysis, we inserted the human type C adenovirus 13*S* mRNA coding region into a plasmid vector, pAS1, designed to express foreign proteins at high levels in *Escherichia coli* (*11, 12*). This vector utilizes efficient regulatory signals derived from phage λ, and expression from the vector is tightly regulated by temperature induction. Cells are grown to high density at low temperature without expression and then induced at elevated temperature to express the gene product rapidly and efficiently. The pAS1 vector allows precise fusion of the foreign coding sequences directly to the translation initiation signal carried on the plasmid. A recombinant plasmid, pJN20, containing an intact, full-length DNA copy of the E1a coding sequence (Fig. 1A), was constructed (*13*). The resultant Ad2-Ad5 hybrid E1a gene in pJN20 precisely lacks the 13*S* mRNA intron and is functionally indistinguishable from the normal Ad5 or Ad2 E1a gene in its ability to induce early viral transcription (*14*).

Our strategy was to insert the DNA sequence coding for E1a into the plasmid expression vector, pAS1 (*11, 12*), in such a way that its expression was controlled exclusively by transcriptional and translational signals derived from phage λ. We used a multistep approach (*15*) to obtain an in-frame fusion of the translation cation initiation codon on the vector at the third codon of E1a (Fig. 1B). The E1a coding sequence in the resulting vector, pAS1-E1A410, is identical to authentic E1a except for the deletion of the codon for the second amino acid. The pAS1-E1A310 (Fig. 1B) vector serves as a control for all subsequent experiments on expression, identification, and functional analysis of the E1a gene product made in *E. coli*; this vector differs by only 4 base pairs (bp) from the pAS1-E1A410 construct, but because of this 4-bp frame shift, pAS1-E1A310 cannot produce an E1a gene product. Moreover, the pAS1-E1A310 construction retains the unique Bam HI restriction site positioned precisely at the 5′ end of the E1a coding sequence. This site allows ready access to the gene for making additional alterations (deletions, additions, or substitutions) to the gene. In constructing the pAS1-E1A310 vector we removed the second codon of the E1a coding sequences. Previous characterization of a mutant adenovirus containing a deletion at the 5′ end of the Ela coding sequence indicates that the 14 amino acids at the amino-terminal end of the E1a protein are not essential for its activity (*16*).

We examined the rate of synthesis of the E1a gene product in *E. coli* by labeling cells transformed with either pAS1-E1A410 or pAS1-E1A310 for 90 seconds

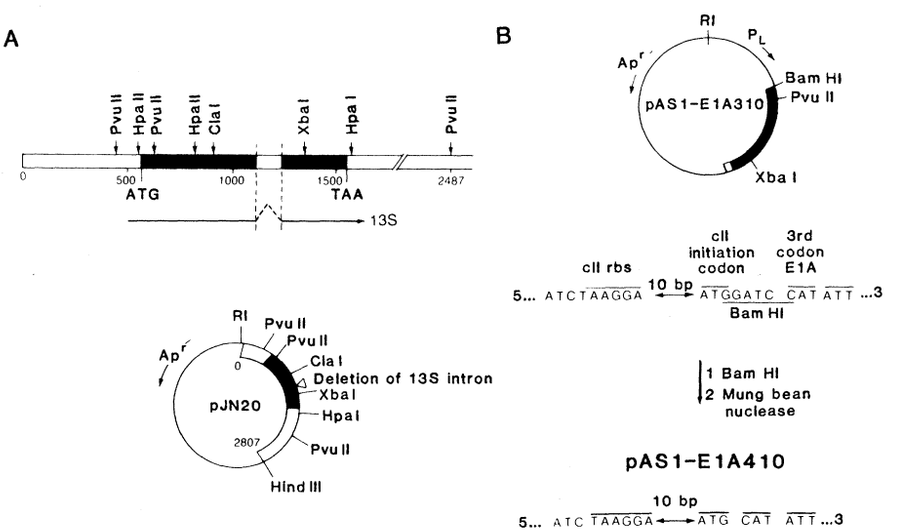

Fig. 1 (A) The adenovirus E1a region and structure of plasmid pJN20. (Top) Genomic map of the left end of the human type C adenovirus (Ad2 or Ad5) genome, extending from the left end (0) to the Pvu II site at nucleotide position 2487. The solid thick line represents the coding region for the 289–amino acid product of the E1a 13*S* mRNA. The initiation (ATG) and termination (TAA) codons for E1a are indicated. The intron of the 13*S* mRNA removed by RNA splicing and the structure of the resulting 13*S* mRNA are also indicated. (Bottom) Schematic drawing of plasmid pJN20, constructed as described (*13*). The thin single line represents pBR322 sequences and the double line represents adenovirus sequences extending from the left end (0) to the Hind III site at nucleotide position 2807. The solid thick line represents the E1a coding sequence from which the intron of the E1a 13*S* mRNA has been precisely removed. Ap^r indicates the β-lactamase gene. Restriction sites referred to in the text are shown. (B) Schematic diagram of plasmid pAS1-E1A310 and construction of plasmid pAS1-E1A410. The details of the construction of pAS1-E1A310 and pAS1-E1A410 are described (*15*). The thin single line represents sequences from the expression vector pAS1 (*11*). Transcription initiates at the bacteriophage λ promoter, P_L, in the direction indicated. The double line represents adenovirus sequences extending from a Bam HI site introduced at the 5′ end of the E1a gene (solid thick line) to the Hpa I site just beyond the E1a coding sequence. The nucleotide sequence surrounding the Bam HI restriction site (that is, the fusion junction between pAS1 and E1a) in pAS1-E1A310 is shown. The *cII* ribosome binding site (*rbs*) and translation initiation codon are indicated, as is the out-of-frame fusion with the E1a coding sequence. The E1a coding sequence was positioned in-frame with the initiation codon to yield pAS1-E1A410 (*15*).

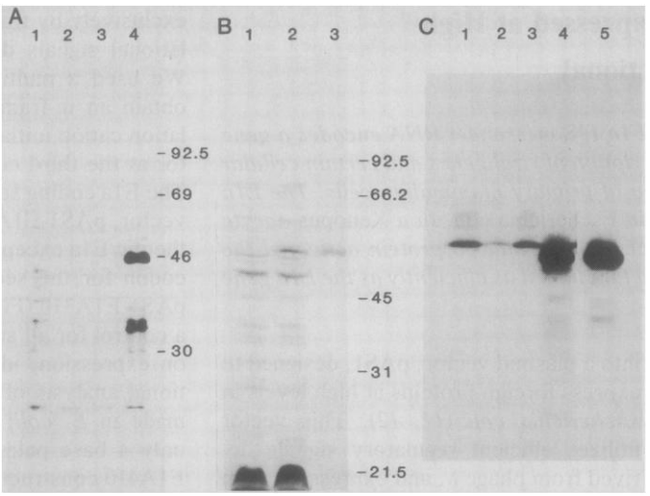

Fig. 2. (A) Expression of E1a in *E. coli* detected by pulse-labeling. *Escherichia coli* strain N5151 (c1*ts*857) (*11*) carrying pAS1-E1A310 (lanes 1 and 2) or pAS1-E1A410 (lanes 3 and 4) were pulse-labeled with [^{35}S]methionine (*11*) for 90 seconds before temperature induction (lanes 1 and 3) and at 30 minutes after temperature induction (lanes 2 and 4). The labeled proteins from total cell extracts were resolved by SDS-PAGE (12.5 percent acrylamide) in one dimension and visualized by fluorography. The position and molecular size (in kilodaltons) of markers (Amersham) are indicated. (B) Accumulation of E1a protein in *E. coli*. *Escherichia coli* strain N5151 carrying pAS1-E1A410 was grown to an optical density at 650 nm of 0.8 and then temperature-induced for expression as described earlier (*26*). Total cell extracts were prepared from cells obtained before induction (lane 1) and at 90 minutes after induction (lane 2). Cells were sampled in 1-ml portions, centrifuged, suspended in lysis buffer (10 m*M* tris HCl, *p*H 7.5 at 25°C, containing 10 percent glycerol, 5 percent 2-mercaptoethanol, 2 percent SDS, and 0.1 percent bromphenol blue), and incubated on a boiling water bath for 5 minutes. Proteins were analyzed by SDS-PAGE (15 percent acrylamide) and were visualized by staining with Coomassie brilliant blue R-250. Lane 3 contains 8 μg of purified E1a protein (*23*). The protein concentration was measured by the method of Bradford (*27*), with bovine serum albumin used as a standard. Molecular size markers (Bio-Rad) are indicated. (C) Detection of E1a synthesized in *E. coli* by immunoblot analysis. E1a protein synthesized in *E. coli* was detected by Western blot analysis (*17*) with an E1a peptide–specific antiserum (*17*). Total cell extracts were prepared from *E. coli* strain N5151 transformed with either pAS1-E1A310 (lanes 1 and 2) or pAS1-E1A410 (lanes 3 and 4). Total cell extracts were prepared before temperature induction (lanes 1 and 3) and 60 minutes after temperature induction (lanes 2 and 4) of E1a expression, and the proteins were resolved by SDS-PAGE (12 percent acrylamide). Lane 5 contains 60 ng of purified E1a protein (the product of pAS1-E1A410).

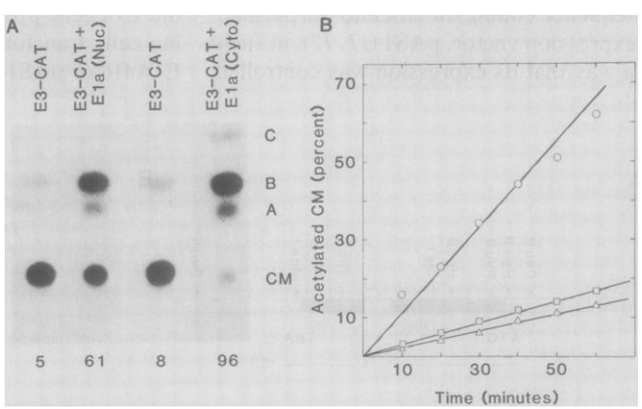

Fig. 3. Activation of the adenovirus E3 promoter by purified E1a protein microinjected into *Xenopus* oocytes. (A) Thin-layer chromatographic assay showing the level of CAT activity in extracts from *Xenopus* oocytes receiving microinjections. Oocytes were obtained and microinjected as described earlier (*22*). When individual oocytes received two injections, the oocytes were incubated in Barth's medium (*28*) for 1 hour between injections. After microinjection, the oocytes were cultured in Barth's medium at 19°C for 30 hours preceding the CAT assay. The CAT assay was performed as described (*13*) by monitoring by the conversion of [^{14}C]chloramphenicol (*CM*) to its acetylated forms (A, B, and C). All oocytes used in the assay were obtained from the same female, and the same oocyte equivalent amount of extract was assayed. Extract was prepared from 15 oocytes for each assay. The E3-CAT gene (0.5 ng of pKCAT23) (*3*) was microinjected alone into the nucleus of the oocyte (first and third lanes) or the E3-CAT gene was injected along with purified E1a protein (0.5 ng; see Fig. 2B) (second lane). In some cases the oocytes received two microinjections. The E3-CAT gene was first injected into the nucleus and then purified E1a protein was injected into the cytoplasm (lane 4). The number below each lane indicates the percent conversion of chloramphenicol to its acetylated forms, as quantified by liquid scintillation counting. (B) Graph showing the levels of CAT activity in various extracts obtained from *Xenopus* oocytes. Data are as the percentage of chloramphenicol converted to acetylated forms as a function of time. Microinjection into oocytes and the CAT assay were performed as described above. The E3-CAT gene (0.5 ng of pKCAT23) was injected into the nucleus of each oocyte. The cytoplasm of the same oocytes was then injected with (○) purified E1a protein (10 ng) derived from pAS1-E1A410 or (△) an extract prepared from *E. coli* transformed with pAS1-E1A310; (□) was not injected.

(pulse-labeling) with [^{35}S]methionine (*11*) before and after temperature induction of E1a expression. A major new protein was synthesized in response to the induction (Fig. 2A). This product was induced selectively from the pAS1-E1A410 vector and was not produced from the pAS1-E1A310 control vector. Comparison with marker proteins indicates that the major product has an apparent molecular size of about 47 kilodaltons (kD) as estimated from its mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). This protein presumably represents the full-length translation product of the E1a gene in *E. coli*. A second, relatively minor product with a molecular size of approximately 33 kD was also induced from the pAS1-E1A410 vector (Fig. 2A). Pulse-labeling and pulse-chase experiments, with pAS1 derivatives that encode and express E1a variants with amino-terminal or carboxyl-terminal deletions (or both), have shown that the 33-kD product is a truncated form of the E1a protein, which appears to be lacking a substantial portion of the carboxyl-terminal region of the gene (data not shown). This 33-kD E1a derivative apparently arises from premature translation termination. Pulse-chase data show that the 33-kD E1a derivative is not stable in *E. coli* and, indeed, this protein does not accumulate in *E. coli* or copurify with the full-length 47-kD product (see below). The pulse-labeling data indicate that, after induction, more than 90 percent of the protein synthesis occurring in the bacteria is directed toward the synthesis of E1a protein. Thus, after temperature induction, the bacterial cell appears to devote a major proportion of its synthetic capacity to the expression of the E1a gene product.

The high rate of synthesis of the E1a protein in *E. coli* resulted in significant levels of E1a accumulation. Figure 2B shows a Coomassie-stained SDS-PAGE gel pattern of proteins present in total cell extracts of *E. coli* carrying the pAS1-E1A410 vector. After induction, a major new protein having the appropriate molecular size was seen. The SDS-PAGE mobility of this protein varies considerably, depending on the electrophoretic conditions; the corresponding apparent molecular size ranges from 45 to 55 kD (for example, compare Fig. 2A and Fig. 2B). Indeed, when run on the same SDS-PAGE gel, the induced Coomassie staining band and the major induced pulse-labeled band migrated exactly together (data not shown). Between 1 and 2 hours after induction, the E1a protein is a major constituent in the cell, accounting

for about 5 percent of the total cellular protein as judged from its relative staining intensity in the SDS-PAGE gel (Fig. 2B).

To confirm the identity of the Coomassie-stained 45- to 55-kD SDS-PAGE band as E1a, we used an E1a peptide–specific antiserum (*17*). Immunoblot (Western blot) analysis (*17*) was carried out on total cell extracts prepared from *E. coli* transformed with either the control vector (pAS1-E1A310) or the E1a expression vector (pAS1-E1A410). The results (Fig. 2C) show that the induced product made in, and purified from, the bacteria containing pAS1-E1A410 is recognized by the E1a peptide–specific antibody.

The DNA sequence of the E1a gene predicts a protein product of 289 amino acids with a calculated molecular weight of 32 kD (*18*). Yet, the major product of this gene as it is made in *E. coli* has an apparent molecular weight, based on SDS-PAGE mobility, ranging from 45 to 55 kD (that is, 13 to 23 kD larger than its expected size). A similar size anomaly was observed for the E1a protein produced in adenovirus-infected human cells when E1a peptide–specific antisera were used for detection of E1a (*17, 19*). Products of similar SDS-PAGE mobility were observed in vitro when E1a-specific mRNA was translated in cell-free systems (*20*). Since the E1a protein produced in *E. coli* shows the same anomalous SDS-PAGE mobility, we conclude that this feature is an intrinsic property of the E1a protein and does not result from posttranscriptional or posttranslational modifications specific to the eukaryotic cell. We suspect that the aberrant mobility of the E1a protein in SDS-PAGE results from some structural feature imposed by its high proline content (16 percent). This atypical characteristic suggests that the native structure of E1a may also be quite unusual.

To test the functional activity of the E1a protein produced in *E. coli*, we purified the protein and examined its ability to activate transcription from the E1a-responsive adenovirus promoter, E3. The E1a protein was purified to near homogeneity (Fig. 2B) with a yield of about 1 mg of purified E1a protein per gram (wet weight) of induced *E. coli* cells. A detailed description of the protein purification procedure will be given elsewhere (*21*). A plasmid vector, pKCAT23 (*3*), which carries the E3 promoter fused to the chloramphenical acetyltransferase gene (CAT), was used to examine E1a function in a *Xenopus* oocyte microinjection assay (*22*). This same assay system was used previously to demonstrate that, in the presence of the E1a gene, CAT expression from pKCAT23 was increased up to eightfold (*22*). We performed similar experiments except that pure *E. coli*–made E1a protein, rather than DNA, was microinjected along with the E3-CAT fusion vector. The results of these experiments are shown in Fig. 3. Injection of pure E1a protein reproducibly induced up to a tenfold increase in CAT expression. An *E. coli* extract prepared in the same way from cells carrying the out-of-frame pAS1-E1A310 construction was injected into *Xenopus* oocytes to serve as a control. These cells do not express E1a and, as expected, no induction of CAT activity was observed (Fig. 3B). Thus, the *E. coli*–produced E1a protein appears to be at least as effective as the E1a gene itself at inducing CAT expression from pKCAT23.

The E1a protein was effective at E3-CAT induction whether it was injected into the cytoplasm or the nucleus of the oocyte (Fig. 3A). Separation of the oocyte into cytoplasmic and nuclear fractions and analysis of each with an antibody to the pure *E. coli*–produced E1a protein showed that E1a moves rapidly (within 30 minutes) from the cytoplasm to its presumed site of action within the nucleus (data not shown). This nuclear localization is evidently an intrinsic property of the E1a protein. More recent experiments (*21*) show that when pure *E. coli*–produced E1a is microinjected into the cytoplasm of somatic cells (for example, monkey kidney cells), the protein is rapidly taken up and localized in the nucleus. In both oocyte and somatic cell types, this nuclear localization is maintained for many hours. Apparently, the information required for its nuclear location must be contained within the native, mature structure of the molecule and does not require active protein synthesis or any other mechanism coupled to nascent polypeptide formation.

Our results demonstrate directly that it is the protein product of the E1a 13*S* mRNA that positively regulates expression from the adenovirus E3 promoter. In addition, our results show that a nucleus-localized, eukaryotic transcription-regulatory protein can be produced in *E. coli* and retain its biological activity when it is microinjected into a eukaryotic cell.

We have purified several variants of the E1a protein produced in *E. coli* (*23*). The availability of E1a and its various derivatives will be useful in examining the effects of structural alterations on E1a function and nuclear localization. In addition, the ability to produce large amounts of E1a protein and its various derivatives will allow us to begin a detailed biochemical and physical characterization of the protein.

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13. Construction of plasmid pJN20 (*29*): pJN20 was derived from plasmid pJOLC3, which contains the Hind III restriction fragment from the left end of the Ad5 genome (map units 0 to 7.8) inserted at the Hind III site in pBR322 (*14*). The Cla I–Xba I DNA segment contained within the Ad5 genomic Hind III fragment in pJOLC3 was replaced with the corresponding Cla I–Xba I fragment obtained from a complementary DNA clone of the E1a 13*S* mRNA from Ad2 (clone 131) (*8*). This construction precisely removed the intron of the 13*S* mRNA from the E1a gene and resulted in an Ad5-Ad2 hybrid gene with Ad5 sequences 5′ to the Cla I site and 3′ to the Xba I site and with Ad2 sequences between the Cla I and Xba I sites. This Ad2-Ad5 hybrid E1a gene differs from the normal Ad5 gene at two codon positions (methionine replaced by leucine at codon position 209, and alanine replaced by valine at codon position 210) and from the normal Ad2 gene at one codon position (serine replaced by proline at codon position 276) (*8*).
14. N. Jones, unpublished data.
15. Construction of plasmid pAS1-E1A310: The 264-bp Hpa II restriction fragment, which contains the 5′ end of the E1a coding sequence (Fig. 1A) was blunt-ended with DNA polymerase (Klenow) and inserted at the Sma I site of pDS26 (*24*) to yield pE1A100. In this way a unique Sma I (Xma I) site is created at the Hpa II site located 10 bp 5′ to the start of the E1a coding sequence.

In pE1A100, the E1a DNA insert is so oriented that a unique Bam HI restriction site occurs upstream of this Sma I site. The pE1A100 was cut with Xma I and treated with Bal31 exonuclease (*25*). The reaction was stopped at several time points (15, 30, 45, and 60 seconds), and the extent of exonuclease digestion was determined by fine restriction. The 45-second Bal31 digestion time point was selected where only 10 to 20 bp had been removed from the end (5′ to the E1a coding sequence) of most of the DNA molecules. The DNA from this Bal31 exonuclease time point was cut with Bam HI, blunt-ended with DNA polymerase (Klenow), and ligated. Ampicillin-resistant transformants were selected and screened for clones in which the Bam HI site was recreated. One of these plasmids, pE1A210, was shown by fine restriction to contain a Bam HI site in which the sixth base of the Bam HI recognition sequence (GGATCC) (G, guanine; A, adenine; T, thymine; C, cytosine) was the first base of the third codon (*CAT*) of the E1a coding sequence. The entire E1a coding sequence was reconstructed in pE1A210 by inserting the 1748-bp Pvu II fragment from pJN20 (Fig. 1) in the correct orientation at the Pvu II site of pE1A210. This procedure yields pE1A310. The entire E1a coding sequence was moved into the expression vector pAS1 by inserting the 895-bp Bam HI–Hpa I DNA fragment (Fig. 1) from pE1A310 between the Bam HI and Nru I sites on pAS1, to yield pAS1-E1A310. The E1a coding sequence in pAS1-E1A310 is not in-frame with the translation initiation signal supplied by the expression vector. Construction of pAS1-E1A410: The vector pAS1-E1A410 was constructed from pAS1-E1A310 by positioning the E1a coding sequence in-frame with the ATG translation initiation signal supplied by the expression vector. This was accomplished by Bam HI restriction of pAS1-E1A310, followed by treatment with calf alkaline phosphatase, limited digestion with mung bean exonuclease (*11*) to remove the four-base 5′ single-strand overhanging ends, and religation of the vector. The correct construction was identified by fine restriction and by expression of the E1a product (detected by pulse-labeling). pAS1-E1A410 encodes a product that is identical to the authentic product of the E1A 13*S* mRNA except for the deletion of the second amino acid.

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30. We thank C. Debouck, Y. Ho, A. Shatzman, and J. Young for many helpful suggestions and discussions, L. Feldman and J. Nevins for providing E1a peptide–specific antiserum, M. Singer for critical reading of the manuscript, and A. Venable for preparation of the manuscript.

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1 February 1984; accepted 24 April 1984

Potentiation of Bleomycin Lethality by Anticalmodulin Drugs: A Role for Calmodulin in DNA Repair

Abstract. *Treatment of exponentially growing Chinese hamster ovary cells with bleomycin causes a dose-dependent decrease in cell survival due to DNA damage. This lethal effect can be potentiated by the addition of a nonlethal dose of the anticalmodulin drug* N-*(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13) but not its inactive analog* N-*(4-aminobutyl)-2-naphthalenesulfonamide (W12). By preventing the repair of damaged DNA, W13 also inhibits recovery from potentially lethal damage induced by bleomycin. These data suggest a role for calmodulin in the DNA repair pathway.*

Many intracellular Ca^{2+}-dependent events are mediated by the Ca^{2+} receptor, calmodulin (*1*). This protein appears to participate in the regulation of cell proliferation by Ca^{2+}, since several anticalmodulin drugs inhibit cell replication (*2–6*), and calmodulin levels appear to change during the cell cycle (*2–4*, *7–9*). We have shown that calmodulin is selectively increased at the boundary of the G_1 and S phases of the cell cycle in exponentially proliferating Chinese hamster ovary (CHO) cells (*2*) as well as on release of growth-arrested cells in response to a mitogenic stimulus (*3*). In both types of proliferative response the entry into and progression through the DNA synthetic (S) phase of the cycle can be specifically and reversibly blocked by the anticalmodulin drug *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13) but not by its inactive analog *N*-(4-aminobutyl)-2-naphthalenesulfonamide (W12). In addition, the antiproliferative effect of interferon is associated with an inhibition in the synthesis of calmodulin that normally occurs at the G_1-S boundary (*10*). These data suggest that calmodulin may be important in the commitment to DNA synthesis. We have examined the effect of W13 on the ability of CHO cells to survive after treatment with the DNA-damaging agent bleomycin. Treatment of exponentially growing cells with bleomycin causes a dose-dependent decrease in cell survival. This lethal effect is potentiated by a nonlethal dose of W13 (or another anticalmodulin drug, trifluoperazine) but not by W12. The anticalmodulin drug W13 also prevents recovery from bleomycin-induced potentially lethal damage by preventing the repair of DNA. Taken together, these data suggest a role for calmodulin in the DNA repair pathway and afford a clue to how cell cycle–dependent changes in the concentration of this ubiquitous Ca^{2+} receptor may regulate cell proliferation.

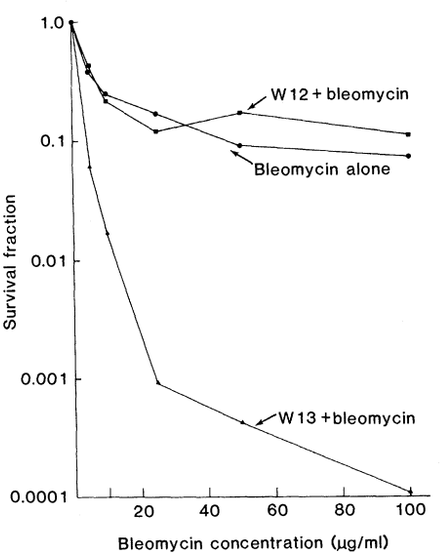

Fig. 1. Dose-response curve for bleomycin and for simultaneous treatment with W12 or W13. Exponentially growing CHO cells were treated for 1 hour with various concentrations of bleomycin (1 to 100 μg/ml) in fresh medium or fresh medium containing W12 or W13 (30 μg/ml). The drugs were then removed, and the cultures were washed twice with Puck's solution A. Cells were immediately removed from the dishes by trypsinization, and survival was determined by colony formation as described (*2*). Cells were immediately plated into replicate culture dishes and propagated in culture for 6 to 8 days to evaluate the ability of the treated cells to form colonies. Colonies were stained with 0.5 percent crystal violet and counted. A cell was considered to have retained reproductive capacity (survival) if it gave rise to a colony of 50 or more cells. The survival index was derived by dividing the number of colonies formed per plate by the total number of single cells initially seeded per plate.

Exponentially growing CHO cells were treated with increasing concentrations of bleomycin for 1 hour in the presence or absence of either W13 or its inactive analog W12 at a dose of 30 μg/ml. This dose of W12 or W13 has no effect on cell survival over a period of 24 hours (*2*). The cultures were then washed free of drug, harvested, and evaluated for cell survival by colony formation. Treatment with increasing concentrations of bleomycin alone results in a biphasic decrease in cell survival (Fig. 1). In the acute phase, almost 80 percent of the cells are killed by a dose of 25 μg/ml. The second phase of the re-