In Vitro Transcription Enhancement by Purified Derivatives of the Glucocorticoid Receptor

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Mammalian glucocorticoid receptors enhance transcription from linked promoters by binding to glucocorticoid response element (GRE) DNA sequences. Understanding the mechanism of receptor action will require biochemical studies with purified components. Enhancement was observed in vitro with derivatives of the receptor that were expressed in *Escherichia coli*, purified, and added to a cell-free extract from *Drosophila* embryo nuclei. Transcription from promoters linked to one or multiple GREs was selectively enhanced by as much as six times. The effect was weaker with only one GRE, and enhancement was abolished by a point mutation that inactivates the GRE in vivo.

THE GLUCOCORTICOID RECEPTOR IS a hormone-dependent transcriptional regulator. Upon binding hormone, the receptor undergoes "transformation," an alteration that enables it to bind to specific glucocorticoid response element (GRE) DNA sequences and to enhance the initiation of transcription by RNA polymerase II from nearby promoters. Although the glucocorticoid receptor is cloned and its multiple functional domains are defined (*1–4*), the precise mechanisms of signal transduction (transformation) and transcriptional enhancement by the receptor are unknown. An understanding of these processes requires that they be recapitulated in vitro with purified components. GRE-mediated transcriptional enhancement in cell-free extracts with purified glucocorticoid receptor protein has not been attained. This could reflect failure to achieve or maintain receptor transformation, inappropriate conditions for transcriptional enhancement, or both.

To overcome these potential problems, we have taken advantage of two findings. First, each of the activities of the intact receptor is conferred by a discrete segment of the protein (*2–4*). This suggests that a study of the transcriptional enhancement activity of the receptor might be facilitated by expressing only those regions that are required for enhancement. Second, GRE-dependent enhancement by the rat glucocorticoid receptor is maintained faithfully when the receptor is expressed in nonmammalian cells, such as *Saccharomyces cerevisiae* (*5*) and *Drosophila melanogaster* (*6*); several regulators and transcription factors that act at RNA polymerase II promoters have similarly been demonstrated to function in distantly related species (*7*). Thus, a highly active cell-free transcription system might be used to achieve receptor-mediated enhancement in vitro, even if the transcription extract is derived from a different species. We obtained glucocorticoid receptor–dependent transcriptional enhancement with the use of truncated receptor derivatives that were expressed in *Escherichia coli*, purified, and added to nuclear extracts from *Drosophila* embryos.

Transcriptional enhancement activity resides within two discrete, noncontiguous regions of the 795–amino acid rat glucocorticoid receptor (Fig. 1A). One of the regions, enh1, is interdigitated with sequences involved in specific GRE binding; its activity can be demonstrated directly with fragments that encompass this region, such as X556, which includes amino acids 407 to 556 (*3*); point mutants within enh1 demonstrate clearly that it is essential for enhancement by the intact receptor (*8*). Specific DNA binding and proper folding of this region is dependent on two Zn (II) ions, each tetrahedrally coordinated with four cysteine sulfur atoms (*9*) to form two "zinc fingers." A second enhancement region, enh2, resides near the NH_2-terminus of the receptor (Fig. 1A). Its activity has been inferred from deletion mutations (*3*) and demonstrated directly by linking segments such as amino acids 106 to 318 to the DNA binding domain of a heterologous protein that itself lacks enhancement activity (*4*).

We constructed a fusion of the enh1 and enh2 segments of the receptor, EX556 (Fig. 1A); this species confers enhancement in a hormone-independent manner and with activity equal to or greater than that of the fully transformed intact receptor in transfected monkey CV-1 cells (*10*). We therefore expressed EX556 in *E. coli* with the T7 promoter–T7 polymerase system (*11*) and purified the protein product, denoted T7EX556, as described for the correspond-

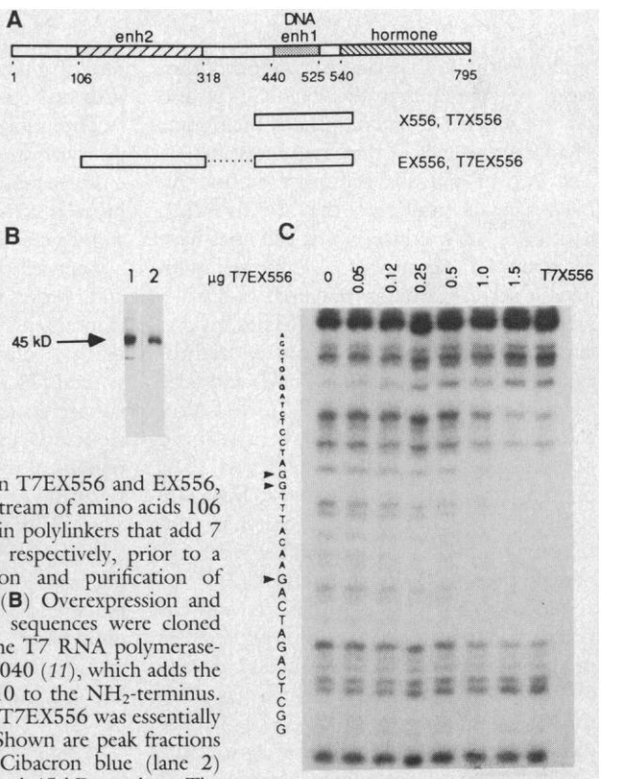

Fig. 1. Expression, purification, and DNA binding activity of glucocorticoid receptor derivatives. (**A**, top) The 795–amino acid coding region of the rat glucocorticoid receptor with the positions of the functionally independent domains for specific DNA binding, hormone binding, and the two regions (enh1 and enh2) that mediate transcriptional enhancement (*2–4*) demarcated. (Bottom) Two receptor derivatives constructed and expressed both in animal cells and in *E. coli*; derivatives expressed by T7 RNA polymerase (see below) in *E. coli* are denoted as T7X556 and T7EX556. T7X556 and X556 contain amino acids 407 to 556; in T7EX556 and EX556, 407 to 556 is fused in-frame downstream of amino acids 106 to 318. The derivatives terminate in polylinkers that add 7 and 16 nonreceptor amino acids, respectively, prior to a termination codon. Overexpression and purification of T7X556 has been described (*9*). (**B**) Overexpression and purification of T7EX556. EX556 sequences were cloned into the unique Bam HI site of the T7 RNA polymerase-dependent expression vector pAR3040 (*11*), which adds the first ten amino acids of T7 gene 10 to the NH_2-terminus. Overexpression and purification of T7EX556 was essentially as described for T7X556 (*9, 12*). Shown are peak fractions from Bio-Rex 70 (lane 1) and Cibacron blue (lane 2) columns that contained the expected 45-kD product. The pooled material from the latter column was used in this study. (**C**) Specific binding of T7EX556 to GRE sequences. Indicated amounts of purified T7EX556 were mixed with a ^{32}P-labeled 256-bp fragment (50 fmol) that contained a GRE site derived from the MTV LTR, incubated at room temperature for 15 min, and then subjected to deoxyribonuclease I footprinting (*24*); 0.75 µg of T7X556 was used. Corresponding DNA sequence is shown on the left. Dimethylsulfate interference sites for binding by the intact receptor are indicated by arrowheads (*16, 25*).

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ing enh1 derivative, T7X556, with minor modifications (*9, 12*) (Fig. 1B). Purified T7EX556 recognized and bound to GRE sequences with characteristics similar to both T7X556 (*9*) and intact receptor from mammalian cells (*13*), as assessed by deoxyribonuclease I footprinting (Fig. 1C).

For our in vitro assays of RNA polymerase II transcription, we used nuclear extracts from *Drosophila* embryos (*14, 15*), which appear to be much more active than the HeLa cell extracts typically used. Receptor activity in the *Drosophila* extracts was tested with a template carrying a chloramphenicol acetyltransferase (CAT) reporter gene driven by the *Drosophila* alcohol dehydrogenase (Adh) distal promoter (*15*), with GRE sequences inserted at −33 bp relative to the transcription start site (Fig. 2). Transcription initiation was measured by primer extension from a CAT-specific oligonucleotide; properly initiated transcripts were 87 nucleotides in length. In addition, a pseudo–wild-type (Ψwt) gene, bearing an Adh promoter and an 8-bp insertion at +53 (*15*), but without linked GRE sequences, was included in each experiment as an internal control; the Ψwt primer extension product was 95 nucleotides.

Transcription was dependent upon the extract (Fig. 2, lane 4) and was inhibited by low levels of α-amanitin (lanes 5 and 8), as expected for transcription by RNA polymerase II. Addition of purified T7EX556 selectively increased by three times the transcription from the GRE-containing test gene, with no effect on Ψwt expression. Similar results were obtained using GRE elements from either the tyrosine aminotransferase (TAT) gene or the mouse mammary tumor virus (MTV) long terminal repeat (LTR) (compare lanes 6 and 7 with lanes 9 and 10). In contrast, enhancement was not observed when the test promoter was linked to a mutant GRE, MTVC13 (lanes 11 and 12), which has a point mutation that abolishes both receptor-DNA binding in vitro and enhancement in vivo (*16*). Enhancement by T7EX556 under these in vitro conditions therefore depended on the presence of a linked functional GRE. Although the magnitude of enhancement is modest in these experiments, the effect was observed in each of >30 experiments. Thus, the *E. coli*–produced T7EX556 receptor derivative is competent to confer enhancement in vitro.

Transcription enhancement increases in transfected *Drosophila* cells when the single GRE element at −33 is replaced by six tandem GREs (*6*), and in animal cells when multimerized GREs are adjacent to the thymidine kinase promoter (*17*). In our in vitro system, six GREs at least doubled the enhancement detected with one GRE and decreased by two-thirds the concentration of receptor required for a maximal response. Thus, slight activation (1.5 times) can be detected with 3 pmol (0.1 μg) of T7EX556 (a 5:1 molar ratio of receptor:GRE), and maximal enhancement (6 times) is achieved with 14 pmol (0.5 μg) (Fig. 3A). In vitro enhancement was GRE-dependent, as Ψwt expression was unaffected; moreover, addition to the reaction of a 36-bp GRE oligonucleotide at molar ratios of 5:1 and 50:1 competitor GRE:test GRE reduced the 6-times enhancement to 1.8 times and undetectable levels, respectively (Fig. 3B).

Both enh1 and enh2 contribute to the overall magnitude of enhancement in vivo (*3, 4, 8*). We obtained similar results in vitro, although we have not yet tested the effects of point mutations in enh1 (*8*). That is, purified T7X556, which includes only the enh1 domain, enhanced transcription by 2.5 times under conditions in which purified T7EX556 enhanced by 6 times (Fig. 3C). We did not observe in vitro enhancement when the GREs were far from the transcription start site. With the GREs at −86 or −262, for example, only a slight effect was maintained, and no stimulation was observed with the GREs positioned at −366 (*18*). In part, this may reflect the modest enhancement that we observe even when the GREs are inserted at −33. Similar difficulties in recapitulating long-range effects in vitro have been reported for the simian virus 40 enhancer and the GAL4 upstream activating sequence (*19*).

In our experiments, the template DNAs were incubated with receptor derivatives for 5 min; extract and nucleoside triphosphates were then added, and the reaction mix was incubated to allow RNA synthesis. Products continued to accumulate for at least 2 hours under these conditions, and the same extent of enhancement was maintained throughout this period (*20*). Thus, both the receptor and the transcription machinery were stable in these extracts during transcription. In addition, the system appeared to be stable

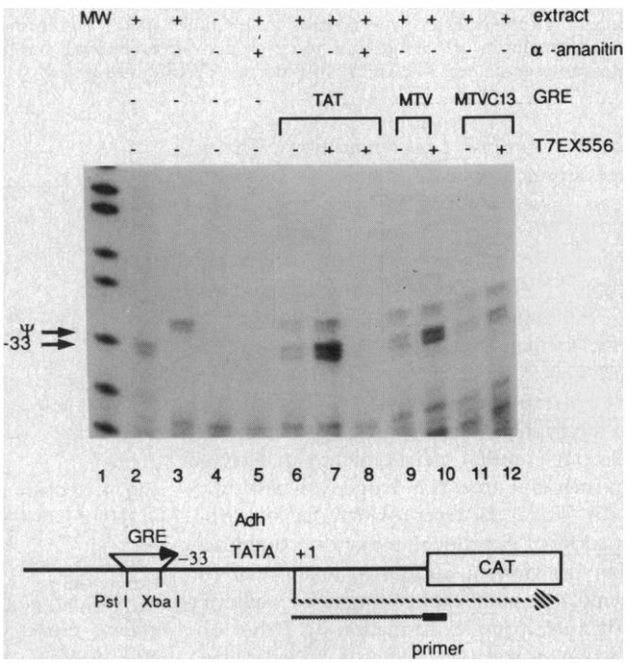

Fig. 2. T7EX556 enhances transcription from a GRE-linked *Drosophila* promoter in *Drosophila* embryo nuclear extract. Transcription was done with plasmid templates that contained single GREs derived either from the tyrosine aminotransferase gene (*26*) (TAT; lanes 4 to 8) or from the MTV LTR (*13, 16*) (MTV; lanes 9 and 10) inserted at the Pst I–Xba I sites (for TAT GRE) or at the Xba I site (for MTV GRE) at −33 of the distal Adh promoter in plasmid pD-33 CAT (band marked −33); the final constructs contain 26-bp or 36-bp GREs, respectively, fused to distal Adh promoter sequences from −33 to +53. MTVC13 (lanes 11 and 12) is an MTV GRE mutant carrying a single base substitution that abolishes specific receptor binding in vitro and GRE activity in vivo (*16*). These constructs were transcribed in the absence (−) or presence (+) of T7EX556. The Ψwt internal control template contains Adh sequences from −86 to +53 with an 8-bp insertion at +53 (*15*) (band marked Ψ). Transcription reaction mixtures (except lane 4) were incubated with *Drosophila* embryo nuclear extract (30 to 45 μg) (*14, 15*), a GRE-containing template (450 ng; 0.12 pmol of GRE), the Ψwt template (130 ng), in the absence (−) or presence (+) of T7EX556 (1.5 μg; 42 pmol); α-amanitin (lanes 5 and 8) was added to 0.5 μg/ml. Reactions (25 μl) were performed in (final concentrations) 25 m*M* Hepes (*p*H 7.6), 3 m*M* DTT, 0.05 m*M* EDTA, 6 m*M* $MgCl_2$, 5% glycerol, 50 m*M* KCl. Templates and receptor were added, incubated for 5 min at 25°C, then extract was added. Transcription was initiated by addition of ATP, TTP, CTP, and GTP (each to 0.6 m*M*) and proceeded for 30 min at 25°C; reactions were stopped by addition of stop mix [75 μl; 200 m*M* NaCl, 20 m*M* EDTA, 1% SDS, and transfer RNA (100 μg/ml)]. RNA was isolated and analyzed by extension of a 27-nucleotide primer complementary to sequences that encompass the CAT initiation codon. Primer extensions were as described (*24*), except that 0.55 μ*M* dNTPs (final) and 50 units of Mo-MLV reverse transcriptase (Bethesda Research Labs.) per reaction were used, and extension was at 42°C for 30 min. Following precipitation with 0.45*M* ammonium acetate and ethanol, pellets were separated on an 8% polyacrylamide, 8*M* urea gel at 40 mA. Products from each reaction were quantitated by excising appropriate bands from the gel, measuring radioactivity in a liquid scintillation counter, and normalizing the −33 band to the Ψwt internal control.

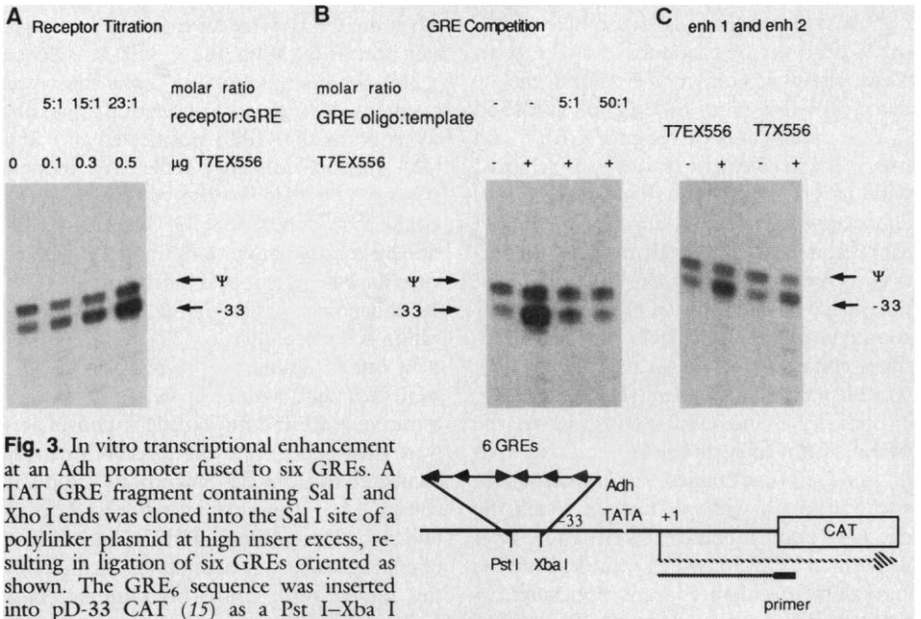

Fig. 3. In vitro transcriptional enhancement at an Adh promoter fused to six GREs. A TAT GRE fragment containing Sal I and Xho I ends was cloned into the Sal I site of a polylinker plasmid at high insert excess, resulting in ligation of six GREs oriented as shown. The GRE_6 sequence was inserted into pD-33 CAT (*15*) as a Pst I–Xba I fragment to create −33 GRE_6. Transcription, primer extension, and quantitation were as in Fig. 2. (**A**) Receptor titration. Reactions were as in Fig. 2, but with 0.1, 0.3, and 0.5 μg of purified T7EX556. (**B**) GRE competition. Receptor-containing (+) reactions included T7EX556 (0.5 μg) in the absence and presence of a 36-bp fragment with a single GRE derived from the MTV LTR (*13, 16*) at molar ratios of 5:1 and 50:1 oligo GRE:template GREs. Quantitation of these results (Fig. 2) revealed that enhancement decreases from 6 times to 1.8 times at 5:1 and is undetectable at 50:1. (**C**) In vitro enhancement by glucocorticoid receptor derivatives bearing one or two enhancement domains. Reactions contained T7EX556 (14 pmol) or T7X556 (28 pmol).

under nontranscription conditions. That is, enhancement was maintained when the extract was incubated with the DNA-T7EX556 mix for 30 min in the absence of nucleoside triphosphates, after which precursors were added to allow synthesis (Fig. 4, experiment I). In contrast, little or no enhancement was observed when the templates were first incubated with extract for 30 min, and then with T7EX556 and nucleoside triphosphates (Fig. 4, experiment II), although modest enhancement was detected in such experiments at longer reaction times (*20*). Thus, the receptor may facilitate formation of functional initiation complexes. By this view, a 30-min incubation of the templates with extract might be sufficient for stable complex formation (*21*), thus obviating a role for receptor added subsequently; however, this experiment alone does not rule out the possibility that extract proteins simply occlude T7EX556 binding. Kinetic assays and extract fractionation studies are necessary to define in detail the stage of the initiation process at which the receptor has its effect.

We have shown that glucocorticoid receptor derivatives produced in *E. coli* and purified to near homogeneity can enhance transcription in nuclear extracts derived from *Drosophila* embryos. The stimulation observed depended on the presence of one or more functional GREs and appeared to be mediated by the enh1 and enh2 enhancement domains defined in vivo. We have not determined whether the use of receptor derivatives without the hormone binding domain, which normally confers the requirement for hormone-dependent transformation, was crucial to the success of our experiments. In this regard, the endogenous estrogen receptor in *Xenopus laevis* liver extracts reportedly stimulates transcription from an estrogen response element–linked promoter in an estrogen-dependent manner (*22*), but hormone-dependent enhancement in in vitro complementation assays with purified receptor derivatives has not been achieved.

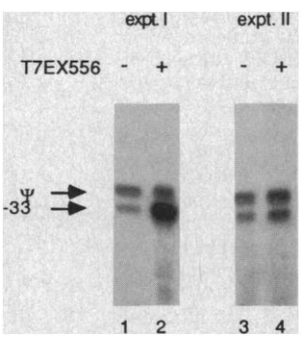

Fig. 4. Order of addition of T7EX556 and nuclear extract to transcription reactions. In experiment I, T7EX556 (0.5 μg) was incubated at 25°C with templates, salts, and buffer for 5 min, embryo extract (45 μg) was added, incubated for 30 min, and then NTPs were added. In experiment II, the embryo extract was first incubated for 30 min with templates, salts, and buffer; T7EX556 (0.5 μg) was added, incubated for 5 min, and NTPs were added. In both experiments, transcription was allowed to proceed for 2 min. RNA was isolated and primer extensions were as in Fig. 2.

Since mammalian receptor derivatives enhance transcription in *Drosophila* extracts, the determinants of transcription initiation and its regulation appear to have conserved their capacity for functional interaction. We may therefore be able to exploit this functional conservation to fractionate, identify, and characterize these components. We have also observed enhancement in HeLa cell nuclear extracts by T7EX556 (*23*), albeit with lower levels of activity. These biochemical strategies should aid in illuminating the mechanisms by which steroid receptors regulate transcription.

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10. The relative magnitude of enhancement by EX556 appears to depend upon promoter context. For example, it had activity similar to that of the hormone-bound intact receptor on the thymidine kinase promoter, whereas it was more active than intact receptor on the β-globin promoter (S. J. Cooper and K. R. Yamamoto, unpublished).
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12. The purification of T7EX556 was as described for T7X556 (*9*) with the following modifications: phenylmethylsulfonyl fluoride (PMSF) (0.1 m*M*) was included in all receptor-containing solutions; 40% $(NH_4)_2SO_4$ was used for precipitation of T7EX556; $ZnCl_2$ was excluded from the dialysis buffer [TEGD50: 50 m*M* tris-HCl *p*H 7.5, 0.5 m*M* EDTA, 10% glycerol, 5 m*M* dithiothreitol (DDT), 50 m*M* NaCl] following $(NH_4)_2SO_4$ precipitation. The dialysate was applied to a Bio-Rex 70 column, and the protein was eluted with a linear 100 to 400 m*M* NaCl gradient. Peak fractions were pooled and loaded onto a Cibacron blue 3GA agarose column, and T7EX556 was eluted with buffer containing 800 m*M* NaCl. Purification was monitored by SDS–polyacrylamide gel electrophoresis; 10% gels were stained with Coomassie brilliant blue, revealing the expected 45-kD product. The Cibacron blue fractions were pooled and dialysed against TGEDZ50 (TGED50 supplemented with 50 μ*M*

$ZnCl_2$). Typical yields obtained were 1 mg of >90% pure T7EX556 per liter of starting culture.
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Monoclonal Antibody–Mediated Tumor Regression by Induction of Apoptosis

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To characterize cell surface molecules involved in control of growth of malignant lymphocytes, monoclonal antibodies were raised against the human B lymphoblast cell line SKW6.4. One monoclonal antibody, anti–APO-1, reacted with a 52-kilodalton antigen (APO-1) on a set of activated human lymphocytes, on malignant human lymphocyte lines, and on some patient-derived leukemic cells. Nanogram quantities of anti–APO-1 completely blocked proliferation of cells bearing APO-1 in vitro in a manner characteristic of a process called programmed cell death or apoptosis. Cell death was preceded by changes in cell morphology and fragmentation of DNA. This process was distinct from antibody- and complement-dependent cell lysis and was mediated by the antibody alone. A single intravenous injection of anti–APO-1 into *nu/nu* mice carrying a xenotransplant of a human B cell tumor induced regression of this tumor within a few days. Histological thin sections of the regressing tumor showed that anti–APO-1 was able to induce apoptosis in vivo. Thus, induction of apoptosis as a consequence of a signal mediated through cell surface molecules like APO-1 may be a useful therapeutic approach in treatment of malignancy.

CELL SURFACE MOLECULES ARE CRUcial in lymphocyte growth control. Such molecules may function as receptors for growth-stimulating cytokines or be associated with receptors and transmit signals essential for growth regulation. Receptor blockade or removal of the stimulating cytokines can lead to decreased lymphocyte growth. Withdrawal of interleukins slow human lymphocyte growth and finally leads to a characteristic form of cell death called "programmed cell death" or apoptosis (*1*). Apoptosis is the most common form of eukaryotic cell death and occurs in embryogenesis, metamorphosis, tissue atrophy, and tumor regression (*2*). It is also induced by cytotoxic T lymphocytes and natural killer and killer cells; by cytokines like tumor necrosis factor (TNF) and lymphotoxin (LT); and by glucocorticoids (*1, 2*). The most characteristic signs of apoptosis are segmentation of the nucleus, condensation of the cytoplasm, membrane blebbing, and DNA fragmentation into multimers of about 180 base pairs (called a "DNA ladder") (*1, 2*). To analyze mechanisms of lymphocyte growth control and to interfere with the replication of lymphoid tumor cells we raised monoclonal antibodies (MAbs) against cell surface molecules involved in these processes.

We found one MAb (anti–APO-1) that blocks growth and induces apoptosis of SKW6.4 cells (*3*). Anti–APO-1 (IgG3, κ, $K_D = 1.9 \times 10^{-10}$) bound to approximately 4×10^4 sites on the surface of SKW6.4 cells (*4*). It specifically immunoprecipitated an endogenously synthesized protein antigen (APO-1) from SKW6.4 cells which, under reducing conditions, was observed on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) as a main band of 52 kD (Fig. 1). Apart from actin (43 kD), which was nonspecifically precipitated with IgG3, anti–APO-1 specifically immunoprecipitated a minor band of 25 kD. This 25-kD protein might either represent a degradation product or be noncovalently associated with the 52-kD protein.

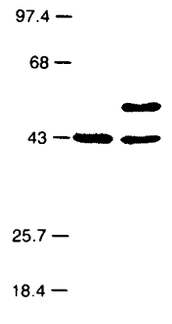

Fig. 1. Molecular weight of the cell surface antigen APO-1: immunoprecipitation of biosynthetically labeled APO-1 from the surface of SKW6.4 cells with either isotype-matched control MAb (left lane) or anti–APO-1 (right lane). The numbers on the left margin indicate the positions of the size markers. Cells (3×10^6) were labeled with 60 μCi of ^{75}Se-labeled methionine (Amersham, Braunschweig, FRG) in 6 ml of methionine-free culture medium (Biochrom, Berlin) for 48 hours. After washing, the cells were incubated in either control MAb or anti–APO-1 (1 μg/ml) at 4°C for 45 min. The cells were washed and resuspended in lysis buffer (tris-buffered saline, *p*H 7.3, 1% Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride, 0.1% aprotinin) at room temperature for 30 min. The lysates were centrifuged and supernatants were incubated with protein A–Sepharose beads (Pharmacia, Uppsala, Sweden) at 4°C for 1 hour. The immune complexes were washed four times with buffer (tris-buffered saline, *p*H 7.3, 0.25% Nonidet P-40) and resuspended in SDS-PAGE sample buffer containing 5% SDS and 5% 2-mercaptoethanol. The samples were heated to 95°C, centrifuged, and counts per minute of the supernatants were determined in a γ-counter. A total of 15,000 cpm were loaded in each lane and analyzed by a 10% SDS-PAGE (*18*). The gel was dried and subjected to autoradiography.

There are two major modes of death in nucleated eukaryotic cells. Necrosis as a result, for example, of complement attack is characterized by swelling of the cells and rupture of the plasma membrane caused by an increase in permeability. Cells that undergo apoptosis, however, show a different biochemical and morphological pattern (*2*). This pattern corresponds to the one induced by anti–APO-1: condensation of the cytoplasm, membrane blebbing (Fig. 2a), and endonuclease-induced DNA fragmentation (*5*) into multimers of approximately 180 bp

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