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ANALYSIS OF POTENTIAL REGULATORY EFFECTS ON GENE EXPRESSION OF ALU-LIKE REPETITIVE DNA FROM THE 5' FLANKING REGION OF THE BOVINE PARATHYROID HORMONE GENE

BY

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ANALYSIS OF POTENTIAL REGULATORY EFFECTS ON GENE EXPRESSION OF ALU-LIKE REPETITIVE DNA FROM THE 5' FLANKING REGION OF THE PARATHYROID HORMONE GENE

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A 659 base pair fragment cleaved on both ends by PstI restriction enzyme approximately 900 base pairs upstream of the parathyroid gene was inserted into a vector, pSVe-CAT, containing an SV40 promoter and the chloramphenicol acetyltransferase (CAT) gene. This construction was made in order to investigate a possible regulatory role of the 659 base pair sequence shown to contain Alu-like repetitive sequences (11). In addition to the repetitive DNA, the segment is also remarkably AT rich.

The study of the sequence's effects on expression was performed by transfecting CV-1 cells. The protein extract of cells transfected with plasmid containing the insertion in both orientations and also the protein extract of cells transfected with empty vector was used in an enzymatic assay to determine CAT activity. Products of the CAT catalyzed reaction were then separated using thin layer chromatography (TLC). The TLC containing various forms of carbon-14 labelled chloramphenicol was then exposed to X-ray film. Subsequent scintillation counting of the TLC yielded relative levels of CAT expression.

The investigation determined that insertion of the Pst I/Pst I

fragment containing Alu-like repetitive DNA in either orientaion resulted in no marked increase or decrease in the level of CAT expression as compared to empty vector. These results suggest that the 659 base pair fragment in the context of these constructions does not regulate gene expression in CV-1 cells.

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I would like to express my gratification to Byron Kemper for granting the priviledge of performing my undergraduate research in his lab and also for his inspirational presence. I would also like to express appreciation to Joseph Cioffi for his guidance and untiring patience. I would like to acknowledge Christine Weaver and Pratap Venepally for their contribution to the tissue culture aspect of this investigation. In addition, I would like to thank all of my fellow coworkers for their individual input and experience into my work and for aiding my development in science during this past year.

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LIST OF ABBREVIATIONS

CAT	Chloramphenicol Acetyltransferase
DNA	Deoxyribonucleic Acid
g	Grams
M	Molar
MEM	Minimal Essential Media
mg	Milligram
mls	Milliliters
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
nm	Nanometers
PTH	Parathyroid Hormone
rpm	Revolutions per Minute
sd	Sterile, Deionized
TLC	Thin Layer Chromatograph
tRNA	Transfer Ribonucleic Acid
UV	Ultraviolet

INTRODUCTION

The parathyroid hormone (PTH) gene is involved in extracellular calcium level regulation in the higher vertebrate. In the past most research dealing with the PTH gene involved structure of the protein and mechanisms of its action. Only recently due to the advent of molecular biology has inquiry into the structure and regulation of the nucleotide sequences regulating and encoding the PTH gene become possible.

The structure revealed by these inquiries has shown the PTH encoding sequence to actually code for a larger polypeptide precursor, preproPTH, which is cleaved to form proPTH and finally to functional PTH, and 84 amino acid peptide (1). The structure of the PTH gene is shown in figure 1. Further studies with the PTH gene dealt with the flanking regions of the gene and the possible regulatory roles of these regions. Two regions of the genomic clone lambdaPTH210, a clone which contains the PTH gene and its 3' and 5' flanking regions, was shown to contain repetitive sequences (2). One region was localized in a 659 base pair PstI/PstI fragment on the 5' side of the PTH gene (2). This region was also found to be AT rich (Fig. 2) and possess strong homology with a repetitive DNA found in flanking regions and introns of the bovine proopiomelano cortin gene (2). Such repetitive sequences have for a long time been considered likely candidates for regulatory roles.

The study of regulation in multicellular eukaryotes has for a long time lagged behind the study of regulation in prokaryotes.

This was due to difficulty in obtaining regulatory mutants, isolation and manipulation of genes.

The regulatory systems of prokaryotes and eukaryotes are very different from each other. Prokaryotes are for the most part free-living unicellular organisms which grow and multiply as long as there are appropriate environmental conditions and an adequate supply of nutrients. The regulatory systems of these organisms focus on providing the maximum growth rate under existing conditions. The lack of membrane surrounding the DNA of prokaryotes ensures the availability of DNA to receive signals in the cytoplasm. Control of initiation of transcription regulates the on-off pattern of protein synthesis(3).

Multicellular eukaryotes are known to possess different requirements. The requirements change in different stages of the life cycle for the eukaryotic organism. In a few respects eukaryotic cells have it easier, since during growth and division the environment of the cells rarely changes with time. The encapsulation of the nucleus in a membrane was a great step in evolution and allowed many significant changes in gene organization and regulation.

The emergence of recombinant DNA technology allowed DNA fragments to be cloned. These clones can then be used to study organization and regulation.

Differences between eukaryotes and prokaryotes which possibly are involved in regulation of the gene include only a single polypeptide chain can be translated from a completed mRNA

molecule as opposed to the operon systems in prokaryotes, DNA of eukaryotes is bound to histones and also to many non-histone proteins, a large portion of the DNA in eukaryotes consists of a few base sequences that are repeated hundreds to millions of times occasionally in tandem, a large fraction of the base sequences in eukaryotic DNA is untranslated, DNA segments in eukaryotes possess mechanisms for rearranging DNA segments, and also the presence of introns in eukaryotic genes (3).

There exist techniques which allow the estimation of the number of genes in an organism. This result can then be compared to the DNA content of one cell in order to approximate the fraction of DNA that consists of coding sequences. Stemming from these results are findings which indicate that E. coli contains about 1500 genes. If a typical protein contains 500 amino acids, 3000 bases are needed to encode one protein or 4.5 million bases for 1500 proteins. Since E. coli contains about eight million bases, half of the sequences encode proteins. Similar calculations for Drosophila melanogaster suggest that it possesses twice as many genes as E. coli and twenty times as much DNA, only five percent of DNA of Drosophila melanogaster consists of coding sequences. Mammalian cells show that a typical cell contains six-hundred times as much DNA as E. coli but only twenty times as many genes, 2 percent of mammalian DNA consists of coding sequences. It becomes clear that a huge fraction of the DNA of eukaryotes is devoted to regulation or some other function (4). It would not, therefore, be surprising

to discover that regulatory mechanisms are very complex in this type of cell.

Repeating sequences are found in all eukaryotes except unicellular organisms. The extent to which repeated sequences exist is shown by C_0t analysis. Use of this technique entails extraction of DNA from an organism followed by denaturation and renaturation. Kinetics of renaturation are determined. Because renaturation is dependent on a concentration process, repeated sequences renature more rapidly. Examination of a C0t curve reveals four classes of sequences (4); unique (single copy), slightly repetitive (1-10 copies), middle repetitive (10 to several hundred copies), and highly repetitive (several hundred to several million copies). Unique sequences account for most of the genes. The slightly repetitive class include genes encoding histones and tRNA genes. Middle repetitive sequences are generally not coding sequences and are believed to be active in regulation. Highly repetitive sequences include short sequences in satellite DNA and also larger repeating sequences. There are few different highly repetitive sequences but the number of copies is so great that these sequences account for twenty percent or more of the mass of DNA.

Even though certain repetitive sequences are believed to function in regulation, actual experimental data has been lacking to uphold these beliefs. A recent publication has assigned a particular function to a repetitive sequence (5).

During examination of enhancer-dependent expression of the

rat insulin gene by using a transient transfection assay to measure the formation of insulin through radioimmunoassay, the authors of the publication constructed plasmids which introduced the enhancer elements (SV40 or MSV) at different lengths (4.0, 2.5, and 0.2 kilobases) from the insulin cap site. These were then transfected into CV-1 cells. The extracts were examined forty hours later for insulin levels. Their results came out to be easy detection of insulin when the enhancers were located 2.5 and 0.15 kilobases from the cap site. However, no activation of insulin expression was seen with the enhancer being 4.0 kilobases away. This suggested that a negative regulatory element might be located between 2.5 and 4.0 kilobases upstream from the rat insulin cap site which possibly could interfere with the effect of the enhancer element. This negative regulatory element was eventually traced to a repetitive sequence DNA.

Encouraged by these results the investigators decided to do a second series of experiments involving constructs in which the sequences upstream from the rat insulin gene were introduced into a plasmid expressing CAT. When the rat insulin sequences located 2.5 to 4.0 kilobases relative to the cap site were positioned 3' to the CAT gene, a significant inhibition of gene expression (79% chloramphenicol conversion reduced to 10% conversion) was observed in transient assays.

To determine whether inhibition affected gene expression at the transcriptional level, a separate set of plasmids was constructed employing the human beta-globin gene as a recorder

sequence (beta-globin transcripts are much more stable in eukaryotic cells than CAT). Various sites of nucleotides from the 5' end of the rat insulin gene were positioned on either the 5' or the 3' side of the beta-globin coding sequences and were then examined in the transient assay for beta-globin mRNA production. When the rat insulin sequences were positioned at the 5' end of the transcription unit in the sense or antisense orientation a significant decrease in transcription (by a factor of ten) was observed.

Similar results were seen when this fragment was inserted at the 3' end of the transcription unit in either the sense or the antisense orientation.

This experiment was followed by a demonstration that the silencer effect of the rat insulin upstream sequence required a cis orientation to the transcriptional unit.

In summary the results of their investigation yielded; silencer sequence must bear a cis relationship to the enhanced gene, the silencer functions on both the 5' and the 3' side of the transcribed gene, and in both orientations in either of these positions. The effects of gene transcription of the silencer appear to be opnosite to that of a classical enhancer element.

Possible mechanisms of the silencer may include alteration of chromatin structure, a possible polymerase exit site, promoter occlusion, and possible reduction in total transcriptional activity of a plasmid.

Another recent study (12) detected discrete high molecular

weight RNA transcribed from the long interspersed repetitive element L1Md. This RNA turned out to be of the same strandedness as the open reading frames. Proposals have been made that suggest L1Md is a retroposon with protein-encoding function.

This study showing an actual activity of a repetitive sequence also gave the implication that other repetitive sequences might function in a similar manner. The only way to validate this possibility is to test other repetitive sequences and observe whether or not any such patterns emerge. A perfect candidate for this study seemed to be the PstI/PstI segment on the 5' end of the PTH gene discussed earlier in this section. Reasons for this sequence being the choice include previous work being performed on this sequence resulting in the full sequence of this segment being known. The homology comparisons that have also been performed on this gene lend further weight on this segment being the segment of choice. The easy availability of this gene in the lab where this work was to be carried out along with the PstI cleaved ends presenting a means of insertion into a plasmid further justified the use of this repetitive sequence for the proposed studies.

The reason pSVe-CAT was chosen as the vector was mainly chosen due to its convenient PstI site being present on the appropriate side of the CAT gene. Presence of the CAT promoter also would be a benefit in the expression studies. The accessibility of all the materials necessary for this study along with the feasibility of the experiment being completed in the allotted

time proved to be an irresistible project and the subject of this paper.

Materials and Methods

Materials

Restriction endonucleases were obtained from Bethesda Research Laboratories or New England Biolabs. T4 polynucleotide kinase was provided by Dr. O. Uhlenbeck. T4 DNA ligase was purchased from Promega Biotec. Cesium chloride for large scale plasmid preparations was purchased from Var Lac Oid Chem. Co. Inc. ^{14}C chloramphenicol was purchased from Du Pont. PstI/PstI fragments containing the repetitive sequence were provided by Joseph Cioffi. pSVe-CAT vector was provided by Dr. Christine Weaver.

Bacterial Strains and Media

Plasmids were propagated in E. coli NM 522. One liter of NM 522 was grown in 2XTY. One Liter of 2XTY consists of 16 grams of Bactotryptone, 10 grams of yeast extract, 5 grams of sodium chloride in a final volume of one liter.

Tissue Culture Strains and Media

CV-1 monkey kidney cells were used for transfections. One liter of media consists of 900 milliliters of H20, 1 package of Minimum Essential Medium (CAT. No. 410-1500) purchased from

Gibco Laboratories, filter sterilize, add penicillin, streptomycin, and fungizone also purchased from Gibco Laboratories, and 100 milliliters of Fetal Bovine Serum (Control No. 28k5462) purchased from Gibco Laboratories. This may be stored for up to one month.

Insertion of PstI/PstI Fragment into pSVe-CAT Vector

Insertion of the PstI/PstI fragment containing the repetitive sequence was accomplished by mixing together 1 microliter of 10X ligation buffer, 1 microliter of ligase, 1 microliter of ATP, 0.5 Microliters of DTT (100mM), 2 microliters of BAPped vector (pSVe-CAT), 2 microliters of PstI cut PTHp210 (1.25 micrograms/microliter), and 2.5 microliters of sdH2O. This was incubated for three hours at 10-20 degrees celsius.

Transformation of Competent NM 522 Cells

The transformation of competent NM 522 cells was accomplished by addition of 5 microliters of ligation mixture to 200 microliters of NM 522 competent cells (6). Let sit on ice for 40 minutes. Heat shock at 37 degrees Celsius for 2 minutes. Add 800 microliters of 2XTY, mix, incubate at 37 degrees Celsius for one hour. Place 200 microliters on each of five B-Amp plates and incubate overnight. B-amp plates are poured from 500 milliliters of H2O, 5 grams of Bactotryptone, 4 grams of NaCl,

and 10 grams of Bacto-Agar.

Minipreps

Minipreps consist of growing an overnight culture of cells from a single colony from overnight plates of transformed NM 522 cells. Cells are spun in an eppendorf tube containing 1.5 mls of the overnight cells for 15 seconds. The supernatant is aspirated and the cells are set on ice.

The cells are dissolved in 175 microliters of STET buffer (contains 50mM Tris pH 8.0, 50mM EDTA, 5% Triton X100, 8% Sucrose). 25 microliters of 10 mg/ml lysozyme (dissolved in STET) are added. Boil for 45 seconds and immediately spin for 15 minutes. The pellet is removed and the supernatant is left behind. 200 microliters of isopropanol are added and the mixture is set at -20 degrees Celsius refrigerator for 10 minutes.

Spin for 5 minutes, pour off liquid, wash gently with 95% ethanol, spin briefly, pour off liquid, and vacuum dessicate for 10 minutes. Dissolve the pellet in 160 microliters of sdH2O. Add 40 microliters of a 5X salt buffer and phenol extract with 100 microliters of phenol and 100 microliters of chloroform. Spin for 5 minutes. Extract with 200 microliters alone.

Add 200 microliters of isopropanol to the supernatant. Spin 5 minutes, drain liquid, wash with 95% ethanol, spin briefly, and vacuum dessicate for 10 minutes.

Dissolve pellet in 15 microliters of sdH2O. The DNA is ready for restriction at this time.

Restriction Digests

Restriction digests are performed throughout the investigation in the following manner using a PstI digest as an example.

6 microliters of miniprep plasmid DNA is used, to this 2 microliters of 5X HinCII buffer is added, 1 microliter of PstI, and 1 microliter of RNAse. The mixtures are incubated at 37 degrees celsius for 3 to 4 hours.

Minigel Screenings

Colonies are screened individually for insertion of the 659 base pair insert by performing a miniprep on each colony individually, followed by a restriction digest as described above. This is followed by adding 2 microliters of a loading dye to the restriction digest and loading of the sample on a 1% agarose minigel. The gel is run at 150 milliamps for about 2 hours in a buffer containing ethidium bromide to stain the DNA. A lane is also included containing appropriate length markers. The gel is then photographed under an ultraviolet light. The photograph is then analyzed as required. The analysis usually requires a standard curve based on the molecular weight markers. From the standard curve sample lengths can be determined.

Large Scale Preparation of Plasmid DNA

After appropriate colonies have screened and found possessing the desired inserts the colony is streak isolated and again screened to see the colonies possess the insert. Large scale preparation of the plasmid DNA may now be attempted.

An overnight culture is inoculated in 5 ml of L-Broth plus ampicillin (50 micrograms per milliliter). Preincubate in a shaker at 37 degrees celsius a 2 liter flask containing 250 milliliters of minimal medium M9 (M9 is 6g/liter Na_2HPO4, 3g/liter KH2PO4, 0.5g/liter NaCl, 1g/liter NH4Cl, 15mg/liter CaCl2*2H2O, 250 mg/liter MgSO4*7H2O, 20g/liter casamino acids (Difco), pH 6.85) 28ml L-3roth, 3ml 10% glucose. Add entire 5 milliliters of overnight culture and incubate until the Absorbance at 600nm reaches 0.8 to 0.9. Add 250 mls of L-Broth and 20 ml of 10% glucose, incubate 30 minutes. Add chloramphenicol to a final concentration of 175 micrograms/milliliter. Incubate overnight in shaker (16 hours). Cool the flask in ice-water for 5 minutes with occasional swirling taking care to keep cells on ice .

The cells are spun down at 9Krpm for 10 minutes and are resuspended in fresh tris-sucrose (tris pH 7.4 0.05M, sucrose 25%) solution to a final volume of 10 milliliters. 1 ml of lysozyme solution (10mg/ml) was added to the cell suspension, and it was left at 0 degrees celsius for 5 minutes. 2 mls of 0.50M

EDTA, pH 8.2, was added, and the sample was left at 0 degrees celsius for another 5 minutes with occasional gentle agitation. An equal volume (13 mls) of 10% Triton EDTA (tris pH 7.4 0.05M, EDTA 0.06M, triton X-100 10%) solution is added stepwise in three aliquots and mixed well. The lysate is centrifuged at 47 K rpm for 60 minutes, in order to spin down most of the chromosomal DNA. One-half volume of 30% PEG solution is added to the supernatant of the centrifugation. The solution is made 1.5M in NaCl by adding the proper amount of dry NaCl and mixing well. Plasmid DNA is spun down at 9 K rpm for 15 minutes. 6 mls of tris-EDTA (tris pH 7.4 0.1M, EDTA 0.01M) is added to the pellet and resuspended. The overall volume is measured and exactly 1gm/ml of optical grade cesium chloride is added and resuspended. 0.3 mls of ethidium bromide is also added to this solution. This solution is spun for 24 hours at 45 K rpm at 15 degrees celsius.

Two red bands could be seen at about the middle of the tube under UV illumination. The top layer of the tube would be proteins and lipids and the pellet would be RNA. The lower band is collected by puncturing the side of the tube with a needle. After adding fresh cesium chloride and ethidium bromide the tube is centrifuged for another 24 hours.

Ethidium bromide is extracted from the DNA solution with one-half volume of n-butanol saturated with cesium chloride. After mixing and centrifugation, the n-butanol solution is removed with a micropipet. This procedure had to be repeated at least 4 times to clarify the DNA solution. Then the DNA solution

was dialized against the DNA buffer for 16 to 20 hours at 4 degrees celsius with at least three changes of buffer, and was stored at 4 degrees celsius with a few drops of CHC13 or frozen at -20 degrees celsius without CHC13.

Transfection of CV-1 Cells

Upon completion of the large scale plasmid preparation, transfection of large scale DNA may be attempted.

CV-1 cells are grown to 80% confluency in two 75 centimeter squared tissue culture flasks. The cells are trypsinized with 1 ml of 1X trypsin. The loosened cells in each flask are resuspended with 10 ml minimal essential medium and 10% fetal calf serum. The cells are pooled from each flask and 1ml portions are used for the cell count. Cells are counted in a cell counter. Three million cells are seeded 150 mm dishes in 40 ml of MEM and 10 % fetal calf serum. 20 hours after the cells were added to the dishes (4 hours before transfection) fresh media is added to the cells.

Forty micrograms of DNA are precipitated in ethanol for each transfection. 0.750 mls of sdH20 is added to the pellets to resuspend them. 0.250 mls of 1M CaC12 is added to make the solution 0.25 M in CaC12, mix thoroughly but gently. Place 1 ml aliquots of 2X HBS into 15 ml falcon tubes (2XHBS is 10 mls 10X BSS and 2.5 mls of 1M Hepes, make up to 50 mls with H20, pH to 7.1 with KOH and filter sterilize). Make CaC12 precipitates of DNA by adding dropwise CaC12 and DNA to the 2X HBS with simulta-

neous vigorous shaking. After incubating CaCl2 precipitates for 30 minutes at room temperature the precipitates were resuspended by gentle pipetting.

DNA precipitates were added dropwise in concentric rings. Swirl each plate gently before incubating in CO2 incubator at 37 degrees celsius. Between 8 to 12 hours after transfection the medium was changed.

45 hours after transfection the medium was decanted and the cells were washed once with versene, followed by 1 wash with 5mls of TEN buffer and then 1 ml of TEN was first added to each plate and cells were scraped off with a rubber policeman, and transferred to an eppendorf tube, followed by a 0.5 ml TEN wash to remove all the cells into the tube.

The cells were spun down at 4 degrees celsius for 10 minutes in a microfuge. Resultant pellets were resuspended in 0.2 mls of 0.25 M tris pH 7.8. The cells were thereupon sonicated at 30 % duty cycle and microtip setting of 3 with 6-7 2 second bursts. The sonicated suspensions were microfuged for 15 minutes at 4 degrees celsius and the supernatant collected into fresh tubes.

Supernatants were incubated in a 60 degreee celsius water block (optional for CV-1 cells but was done since it increases CAT activity by a factor of 2 by denaturing thermolabile deacetylase) for 7-8 minutes and again centrifuged for 15 minutes, supernatants were collected and stored at -20 degrees celsius.

Protein Assay

To use the same amount of protein in each CAT assay; the total protein in each extract is determined by Biorad protein assay (7).

Preparation of 0.2X Biorad dye (reagent) requires the dilution of 1 part Biorad reagent with 4 parts H2O. Filter through Whatman #1, reagent is stable for 2 weeks at 4 degrees celsius.

A standard curve must be made using ovalbumin (stock is 10 mg/ml) in 250mM Tris, pH 7.8 (same solvent as solvent for protein extracts). The standard curve consists concentrations having the following values: 200 micrograms /20 microliters, 75 micrograms/20 microliters, 25 micrograms/20 microliters, 10 micrograms/20 microliters, 5 micrograms/20 microliters, and 2 micrograms/20 microliters.

The assay itself consists of mixing 20 microliter samples with 2 milliliters of 0.2X Biorad reagent and vortexing. Readings at an O.D. of 595nm may be taken after 5 minutes but before 1 hour.

CAT Assay

The CAT assay consists of using 25 to 100 micrograms of protein from each extract (8). 100 microliters of 0.25M tris (pH 7.5) is added to each sample. H2O is added to bring the total volume to 150 microliters. 5 microliters of Carbon 14 labelled chloramphenicol is then added. The mixture is then

incubated for 10 minutes at 37 degrees celsius, the incubation is followed with addition of 30 microliters of 10mM Acetyl CoA. This is again followed by a 37 degree celsius incubation for 2 hours.

1 milliliter of chilled ethyl acetate is added and the organic phase is collected. The organic phase is dried in a speed vac. Resuspend the sample in 15 microliters of ethyl acetate and load on TLC. The TLC is run in a 95:5 mixture of chloroform:methanol solvent. X-ray film is then exposed to the unwrapped TLC at room temperature. After a number of days the film is developed and results of the analysis are visualized.

The quantitation of the results entails cutting out the spots which correspond to acetylated chloramphenicol and counting on a scintillation counter.

RESULTS

The series of experiments involving the influence of the Pst I/Pst I repetitive sequence upon regulation of the CAT gene was initiated with insertion of the 659 base pair PstI segment into an appropriate vector, pSVe-CAT (Fig. 3).

The PstI segment containing the repetitive sequence was inserted into the PstI site of the pSVe-CAT vector. Prior to ligation with the PstI insert the vector was digested with PstI in order for the insert to have an available site for integration into the plasmid. Upon successful digest (examined by agarose gel electrophoresis for linearization by PstI cleavage) a ligation was performed in order to integrate the insert into the vector.

The ligation was followed by transformation of NM522 cells with the newly constructed plasmid. Vector without insert was the main component found in the ligation mixture.

The transformed cells were thereupon plated on media incorporating ampicillin to select for cells containing plasmid. All surviving cells at this point contained plasmid, but only a small minority of these contained plasmid with insert. It now became necessary to screen these colonies for those which contained plasmid with the PstI insert. This screening was performed by subjecting a colony to growth in media, mini prep

plasmid extraction, PstI digestion and agarose gel electrophoresis to determine whether the correct 659 base pair insert was present.

Upon analysis of 48 colonies an observation was arrived at that the PstI insert was not phosphorylated. The above procedure was repeated with kinasing of the insert performed prior to ligation. The 24th colony screened revealed the insert (Fig. 4). The new plasmid was assigned the designation of pSVe-CAT#24.

A decision was made to use this newly constructed plasmid, pSVe-CAT#24 along with vector pSVe-CAT without any insert for analysis of CAT expression.

Transfections require 40 Micrograms of DNA. Acquisition of such an amount of DNA necessitated large scale preparation of pSVe-CAT and pSVe-CAT#24 (Table I).

Table I Results of Large Scale Plasmid Preparation

Plasmid	Concentration (mg/ml)
pSVe-CAT	0.45
pSVe-CAT#24	0.76

This plasmid DNA was then used for transfection of CV-1 cells. Forty Micrograms of protein extract from these cells was used for the CAT assays. CAT assay revealed very similar

levels of CAT activity for pSVe-CAT and pSVe-CAT#24 (Fig. 5).

This series of experiments revealed that the techniques were working properly and that the next phase of the investigation may begin. This next phase was to determine whether orientation played any part on the expression of the CAT gene. The acquisition of both orientations now became necessary.

Seventy-two colonies were screened from the previous ligation. Six clones were found with insert (pSVe-CAT#1, pSVe-CAT#12, pSVe-CAT#15, pSVe-CAT#41, pSVe-CAT#68, and pSVe-CAT#72) in addition to pSVe-CAT#24. Distinguishing one orientation from the other was done by restriction enzyme analysis of mini prep plasmids. A restriction map was obtained of the 659 base pair insert (Fig. 6). From this data NcoI was chosen to perform the cleaving. This decision was made on the basis that NcoI is a six cutter (implies that there would be very few sites which could be cut by this enzyme). More importantly NcoI cut only once in this insert and this cut was an off-center cut (yielding a 280 base pair fragment and an 379 base pair fragment). The vector itself, pSVe-CAT, turned out to have only two sites. The off center cut of NcoI would provide a different pattern of banding after an NcoI digest was subjected to agarose gel electrophoresis for each orientation (Fig. 7).

Actual analysis of such an experiment yielded the predicted results (Fig. 8). Five of the seven plasmids with insert had the insert in the same orientation with reference to the CAT gene as in the PTH gene (pSVe-CAT#1, pSVe-CAT#12, pSVe-CAT#15,

pSVe-CAT#24, and pSVe-CAT#72) and two in the opposite orientation (pSVe-CAT#41, and pSVe-CAT#68). One of each orientation (pSVe-CAT#24, and pSVe-CAT#68) was chosen for analysis for effects on regulation of expression on the CAT gene.

This analysis again required the large scale preparation of plasmid DNA. The large scale preparation had the results listed in Table 2.

Table 2 Results of Large Scale Plasmid Preparation

Plasmid	Concentration (mg/ml)
pSVe-CAT	0.39
pSVe-CAT#24	0.34
pSVe-CAT#68	0.20

CV-1 cells were able to be transfected with this plasmid DNA. Results which were obtained from the CAT assay of the protein extract were quantified by cutting out pertinent spots (Fig. 9) of the TLC and counting in a scintillation counter (Table 3).

Table 3 Results of Scintillation Count

Plasmid	Counts	Relative Values
pSVe-CAT	1323	1.00
pSVe-CAT#24	4420	3.34
pSVe-CAT#68	15362	11.61

The data presented here shows some very dramatic results. In order to avoid making hasty proposals based on just one set of data a decision was made to perform another set of transfections and observe whether the results are reproducible. This repetition of the experiment also had the purpose of removing the possibility that experimental error was involved. More than one transfection of each orientation will also be performed.

The DNA from the last large scale plasmid preparation was used for the next series of transfections of which two were performed on each orientation and also on vector without insert. In order to decrease the exposure time of the film to the TLC the amount of protein used in the CAT assay was changed from 40 micrograms to 100 micrograms. This change cut about 4-5 days out of the usual 8-10 day wait on exposure. Scintillation counting of the TLC spots (Fig. 10) was performed as in the previous experiment (Table 4).

Table 4 Results of CAT Assays

Plasmid	Counts	Mean count of each plasmid	Relative Values
pSVe-CAT	7567	5250	1.00
pSVe-CAT	2956		
pSVe-CAT#24	2399	3445	0.66
pSVe-CAT#24	4492		
pSVe_CAT#68	9907	9200	1.75
pSVe-CAT#68	8520		

The results from Table 4 show very different results upon comparison with results from Table 3. The results in Table 4 are believed to possess greater reliability due to the greater proficiency in performing the critical techniques necessary in such analysis gained by the experimenter between the two experiments.

Checking on variance within an experiment was accomplished by performing a second series of CAT assays on the protein extracts from the last series of transfections. CAT assays were performed utilizing 100 micrograms of DNA (Fig. 11), the same amount which was used in the previous CAT assay. Results are listed in Table 5.

Table 5 Results of CAT Assays

Plasmid	Counts	Mean count of each plasmid	Relative Values
pSVe-CAT	5810	3880	1.00
pSVe-CAT	1950		
pSVe-CAT#24	3875	4298	1.10
pSVe-CAT#24	4721		
pSVe-CAT#68	4783	4016	1.04
pSVe-CAT#68	3250		

The results of Table 5 clearly show high correlation with the results in Table 4. Such reproducibility was desired since this investigation would have been invalidated had CAT assays fluctuated within an experiment.

The results from Table 4 show very different results upon comparison with results from Table 3. The results in Table 4 are believed to possess greater reliability due to the greater proficiency in performing the critical techniques necessary in such analysis gained by the experimenter between the two experiments.

Checking on variance within an experiment was accomplished by performing a second series of CAT assays on the protein extracts from the last series of transfections. CAT assays were performed utilizing 100 micrograms of DNA (Fig. 11), the same amount which was used in the previous CAT assay. Results are listed in Table 5.

Table 5 Results of CAT Assays

Plasmid	Counts	Mean count of each plasmid	Relative Values
pSVe-CAT	5810	3880	1.00
pSVe-CAT	1950		
pSVe-CAT#24	3875	4298	1.10
pSVe-CAT#24	4721		
pSVe-CAT#68	4783	4016	1.04
pSVe-CAT#68	3250		

The results of Table 5 clearly show high correlation with the results in Table 4. Such reproducibility was desired since this investigation would have been invalidated had CAT assays fluctuated within an experiment.

Having shown the reliability or rather the consistency of the CAT assays it becomes desirable to confirm the results of the last series of transfections. The accomplishment of this goal is to be performed by performing 3 more transfections on each of the orientations and also on vector alone. Cat assays of these transfections will also be done in duplicate to reaffirm the reliability of the CAT assays.

Prior to the transfections large scale plasmid preparations had to be performed to obtain the large quantities of DNA necessary for so many transfections (Table 6).

Table 6 Results of Large Scale Plasmid Preparation

Plasmid	Concentration (mg/ml)
pSVe-CAT	0.45
pSVe-CAT#24	0.36
pSVe-CAT#68	0.39

Transfections were now performed utilizing this plasmid DNA. Results of the CAT assays were obtained (Fig. 12 and Fig. 13) and quantitated on a scintillation counter.

Table 7 Results of Scintillation Count

Plasmid	Counts	Mean counts	Relative values
pSVe-CAT	449	432	1.00
pSVe-CAT	247		
pSVe-CAT	618		
pSVe-CAT	566		
pSVe-CAT	243		
pSVe-CAT	473		
pSVe-CAT#24	293	230	0.53
pSVe-CAT#24	210		
pSVe-CAT#24	214		
pSVe-CAT#24	163		
pSVe-CAT#68	169	380	0.88
pSVe-CAT#68	127		
pSVe-CAT#68	1251		
pSVe-CAT#68	409		
pSVe-CAT#68	145		
pSVe-CAT#68	149		

DISCUSSION

Consistent results as determined by CAT analysis were consistently arrived at throughout the investigation except for the second series of transfections (Fig. 7 and Table 3). The rebellious results of this series of transfections can probably be attributed to the the techniques being unperfected at the time, experimental error and and possibly even being within statistical error. This second series also contained only a small fraction of the total transfections done in this investigation. Excluding this small minority of transfections it can be seen that the great majority of the transfections arrive at the conclusion that upon insertion of the PstI/PstI segment into the pSVe-CAT vector no noticeable increase or decrease of the level of expression over pSVe-CAT alone is observed.

This repetitive sequence may not have shown regulatory activity for a number of reasons. One of these being the lack of a bovine PTH secreting cell line. A cell which secretes PTH might possess other factors or sequences which might themselves regulate the proposed regulatory activity of the repetitive sequence. Evidence of such sequence regulation has been found (9) in the chromosome of R6K. The chromosome of R6K possesses multiple origins of replication. One of the origins, gamma, is infrequently used in the plasmid and remains silent in certain plasmid derivatives. The inactivation of of the origin is accomplished by a natural origin silencer located right next to

the ori gamma sequence. The silencer can only function in the cis orientation and can only function if downstream of ori gamma. It appears that the silencer initiates an RNA segment that invades ori gamma and turns it off by either disrupting the ori gamma structure or by competing with a primer RNA. Removal of the silencer blocks the synthesis of silencer RNA and depresses the origin. It is possible that similar mechanisms are acting on the repetitive sequence of the PTH gene.

Another possibility for the regulatory sequence not being observed is that in addition to other factors from a PTH secreting cell needing to be present, extracellular signals might also be needed to express the regulatory activity of this cell. Such signals might be regulatory hormones, proteins, peptides, or even certain ions.

Yet another possibility of why this sequence might not be showing regulatory activity in this type of experiment is lack of enhancer being present. In the original paper (5) in which this type of experiment was performed enhancer was present. One of the proposed mechanisms for this silencing was that the repetitive sequence (with silencing activity) somehow functions by its presence in between an enhancer and its gene. Utilizing the silencing segment of the rat insulin gene in a vector lacking enhancer would either disprove the proposed mechanism or lend further weight to the mechanism. In order to exclude this mechanism operating in the repetitive sequence of the PTH gene it would be desireable to perform the expression analysis in

the future utilizing a vector with enhancer present in an appropriate location.

Variation in expression might also be occuring between the three plasmids at such a level which is not detectable due to the high noise level. Such low level expression may turn out to be the way in which fine tuning of expression is performed. This will only be shown upon development of highly sensitive techniques.

One of the problems in using the CAT assay in conjunction with the TLC analysis is that the exposure time of the autoradiogram is often quite lengthy. A recent development (10) introduces a technique which does not require chromatography, HPLC, or autoradiography. The basis of the assay rests on the use of an inexpensive substrate, tritiated acetate, instead of the usual carbon 14 labelled chloramphenicol. The new technique allows quite dramatic savings of time.

Even though this investigation did not reveal any increase or decrease in expression due to the insertion of the repetitive sequence, this study was important in that it did investigate the possibility of the repetitive sequence possessing regulatory activity. The formation of databases based on these types of experiments which either confirm or rule out regulatory functions of non-coding sequences allows generalizations to be made about regulation based on actual data rather than speculations without any experimental basis. This tour de force approach to understanding regulation may seem long and cumbersome but

when combined with conformation studies of the sequences may turn out to be the method by which regulation will eventually be understood.

In conclusion, this investigation accomplished the insertion of PstI/PstI segment into the PstI site of pSVe-CAT vector in two possible orientations. This was followed by the transfection of CV-1 cells. CAT analysis of the protein extract of these cells revealed no marked increase or decrease in the level CAT being expressed in these cells when compared to cells transfected with only vector.

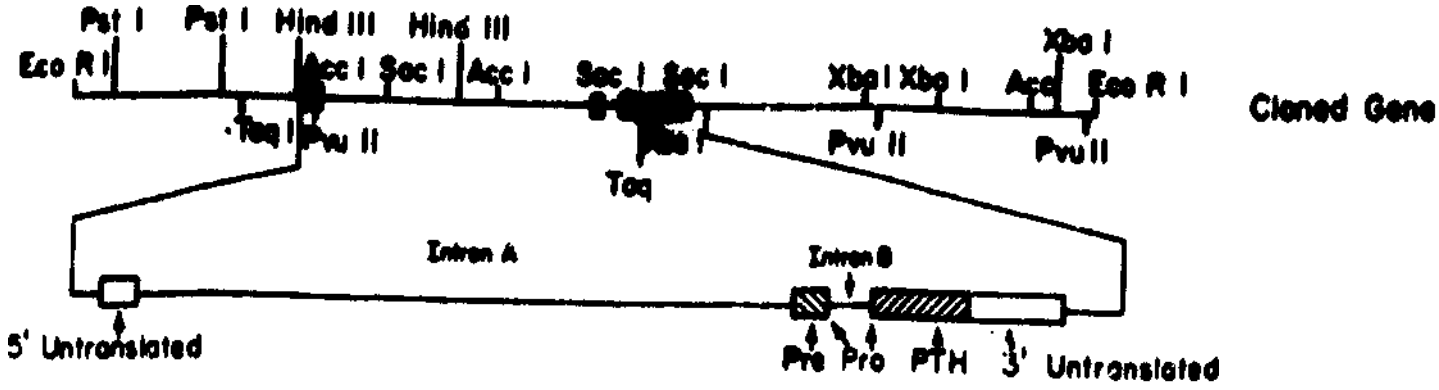


Figure 1. A partial restriction map of the cloned bovine PTH gene showing restriction enzyme cleavage sites relative to the location of the exons (solid black boxes), introns, and flanking regions (reprinted with the permission of Byron Kemper).

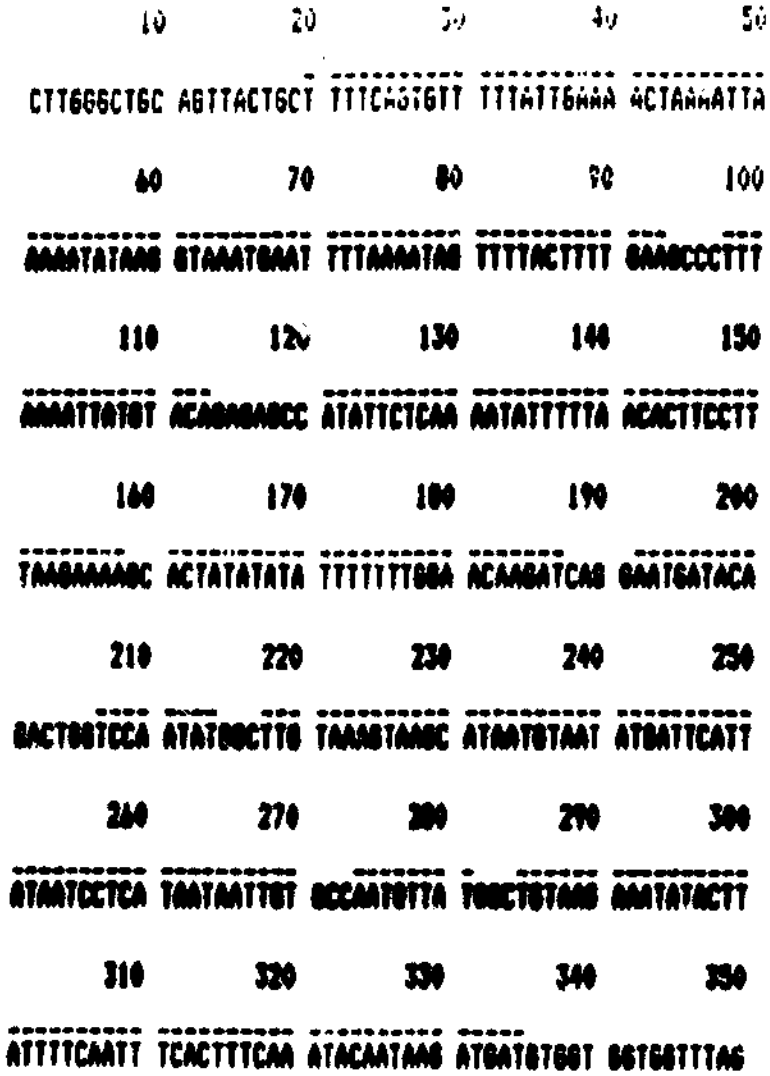


Figure 2. Regions of the PstI/PstI segment that are A-T rich are indicated by (----). These regions were at least 75% A-T using a sliding window of 8 nucleotides (reprinted with permission from Joseph Cioffi).

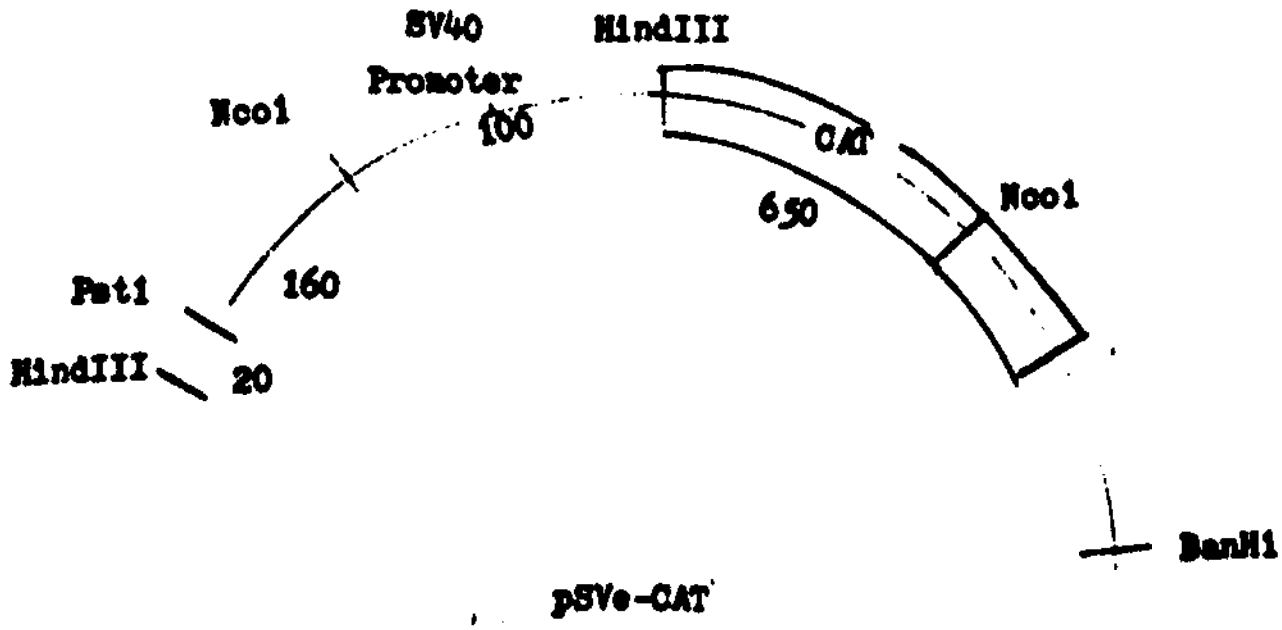


Figure 3. Restriction map of pSVe-CAT vector. Numbers indicate approximate intervening base pairs between restriction sites.

Figure 4. Results of minigel electrophoresis. Left lane is a PstI digest of a plasmid preparation containing the 659 base pair repetitive DNA insertion. The right lane consists of PhiX 174/HaeCut MW marker (from top to bottom number of base pairs represented: 1353, 1078, 872, 603, 310).

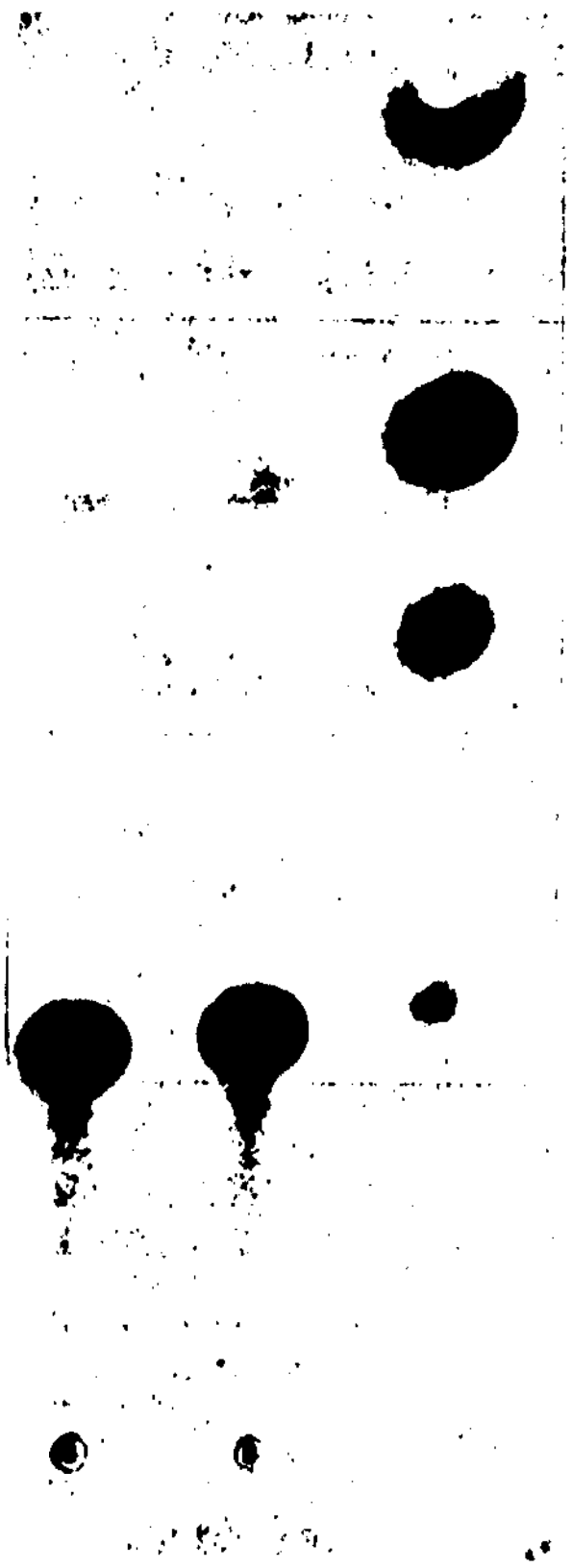


Figure 5. Autoradiogram of CAT assay results. Left lane represents empty vector, middle lane consists of vector with 659 base pair repetitive DNA sequence insertion, right lane is positive control.

Figure 6. Restriction enzyme sites for the 659 base pair repetitive DNA insert (use with permission of Joseph Cioffi).

Restriction Enzyme	Location	Size
Aha III TTTAAA	71	139
	98	27
	591	493
Alu I AGCT	390	520
	397	7
	529	132
Ava II GGA/TCC	205	659
Bbv I GCAGC	650	659
Bst NI CCA/TGG	476	646
	489	13
Dde I CT-AG	353	397
	526	173
	561	35
	615	54
Fnu4 HI GC-GC	6	15
	391	385
	650	259
Hinf I GA-TC	243	389
	413	170
	463	50
	480	17
	513	33
Mbo II GAAGA/TCTTC	416	609
	466	50
Mnl I CCTC/GAGG	256	514
	401	145
Nco I CCATGG	379	659
Pst I CTGCAG	7	18
	648	641
Rsa I GTAC	109	659
Sau 3A I GATC	185	659

(Figure 6 continued)

Sau96 I GG-CC	205	659
Scrf I CC-GG	476	646
	489	13

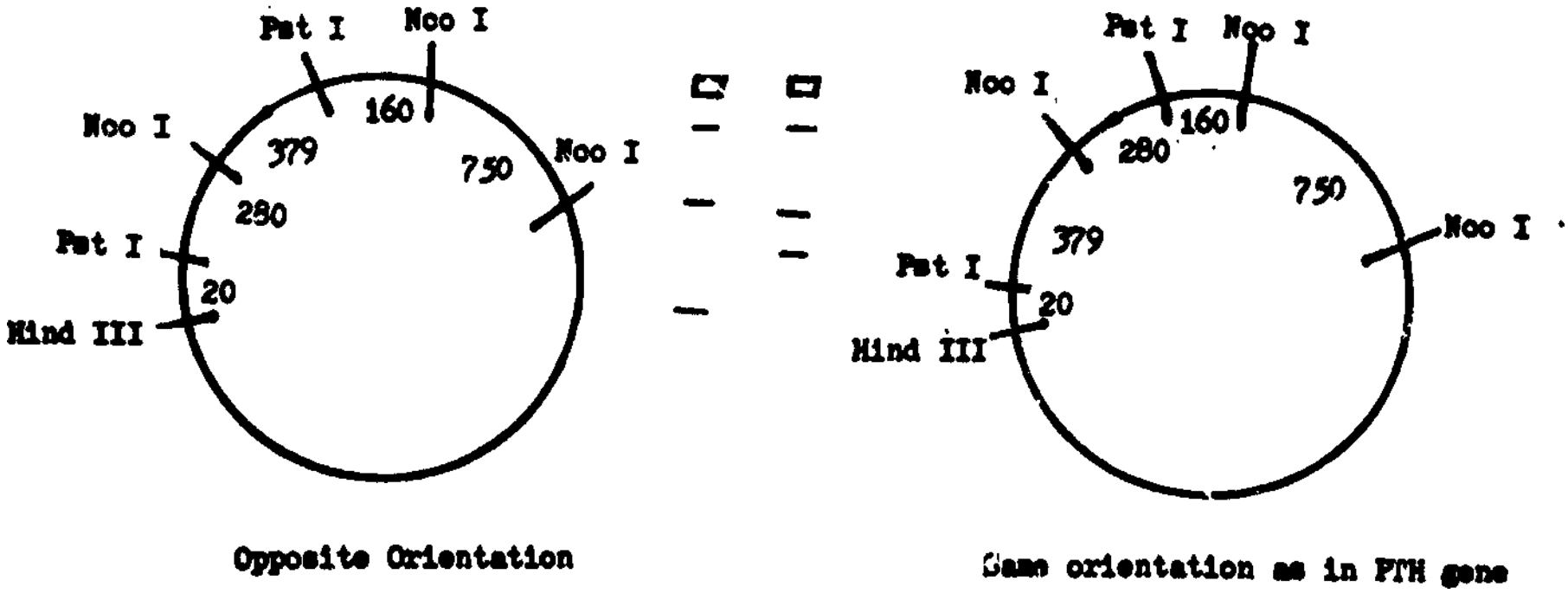


Figure 7. Vector alone and with 659 base pair repetitive DNA sequence in both orientations. Also included is predicted eletrophoresis pattern of the two orientations.

Figure 8. Actual minigel electrophoresis pattern of (from left to right) empty vector, vector with insert in the same orientation as it appears in the upstream region of the PTH gene, and in its opposite orientation.

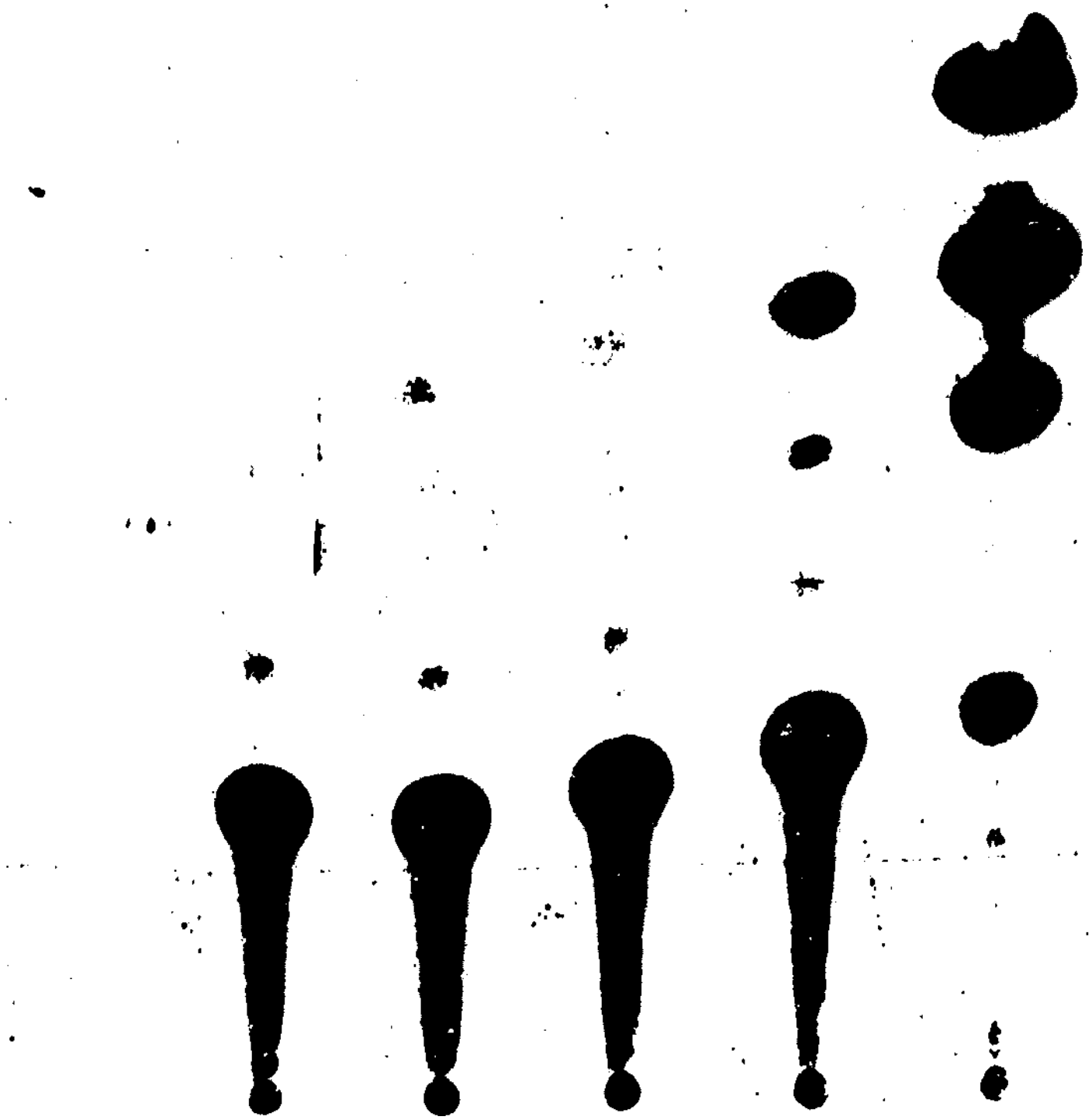
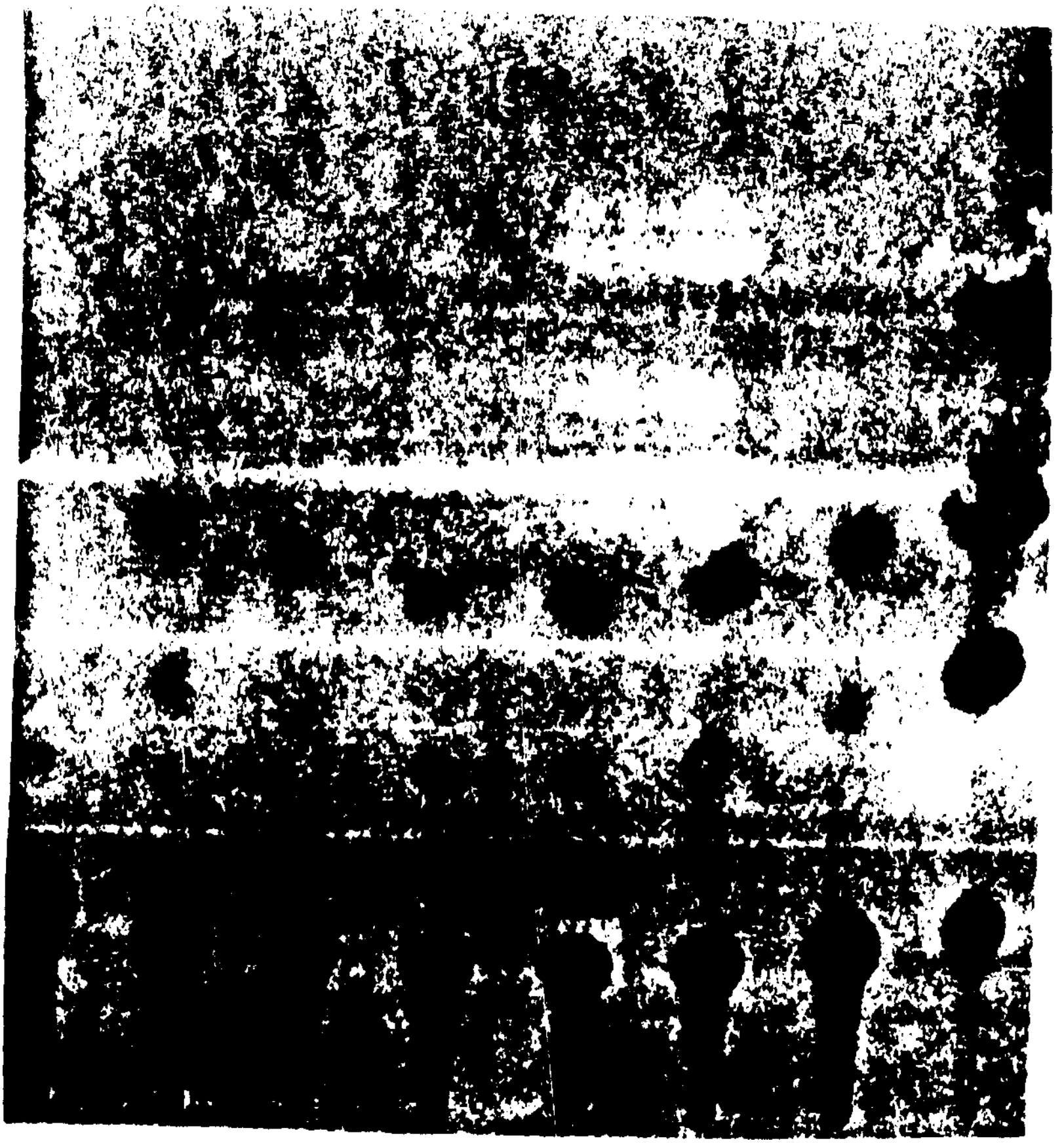


Figure 9. Autoradiogram of second series of transfections (from left to right; negative control, without insert, same orientation as fragment appears in PTH gene, opposite orientation, positive control.



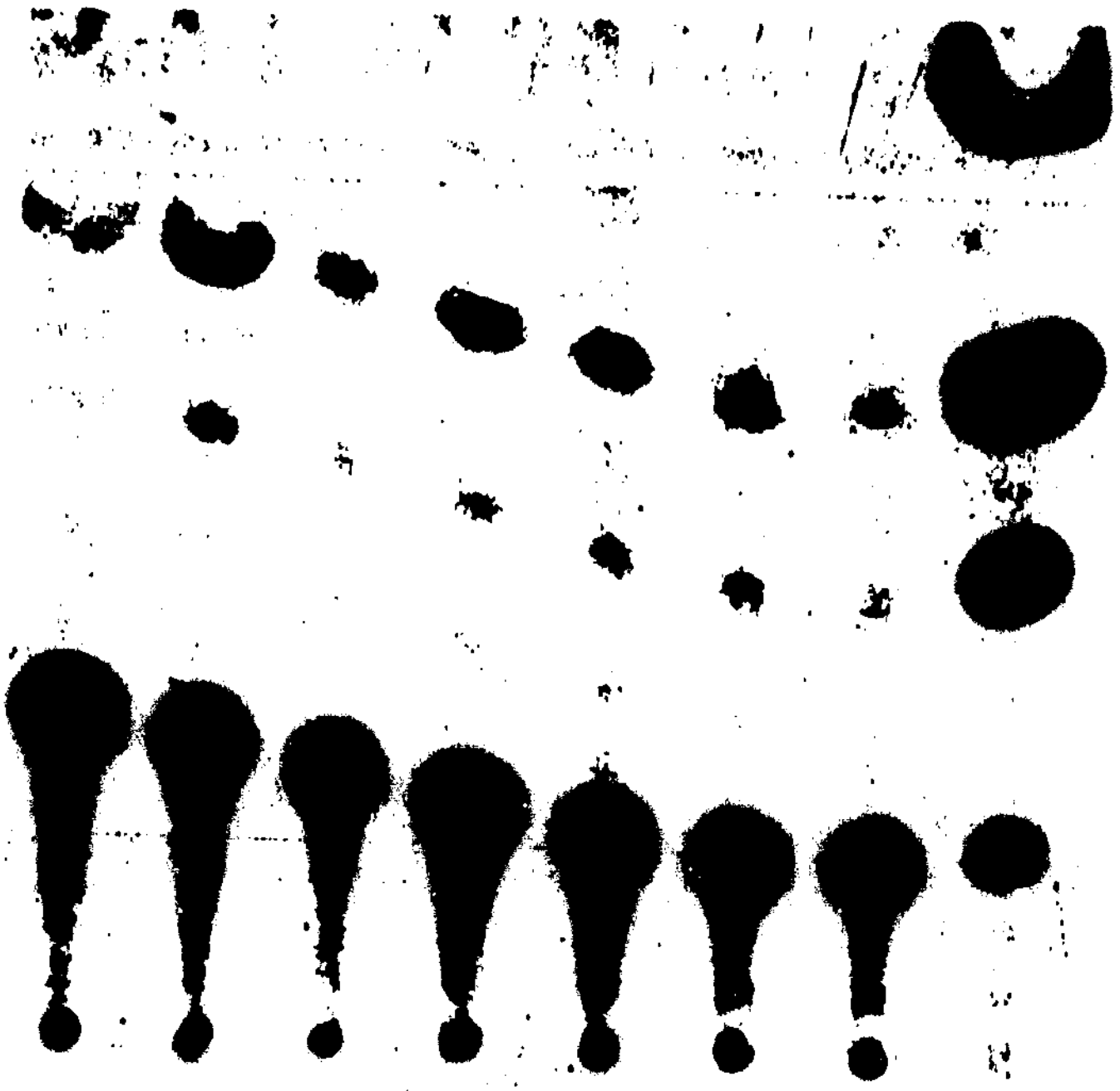


Figure 11. Second series of CAT assays on third series of transfections (from left to right: negative control, pSVo-CAT, [illegible], pSVo-CAT/CA, pSVo-CAT/SA, pSVo-CAT/AA, positive control).

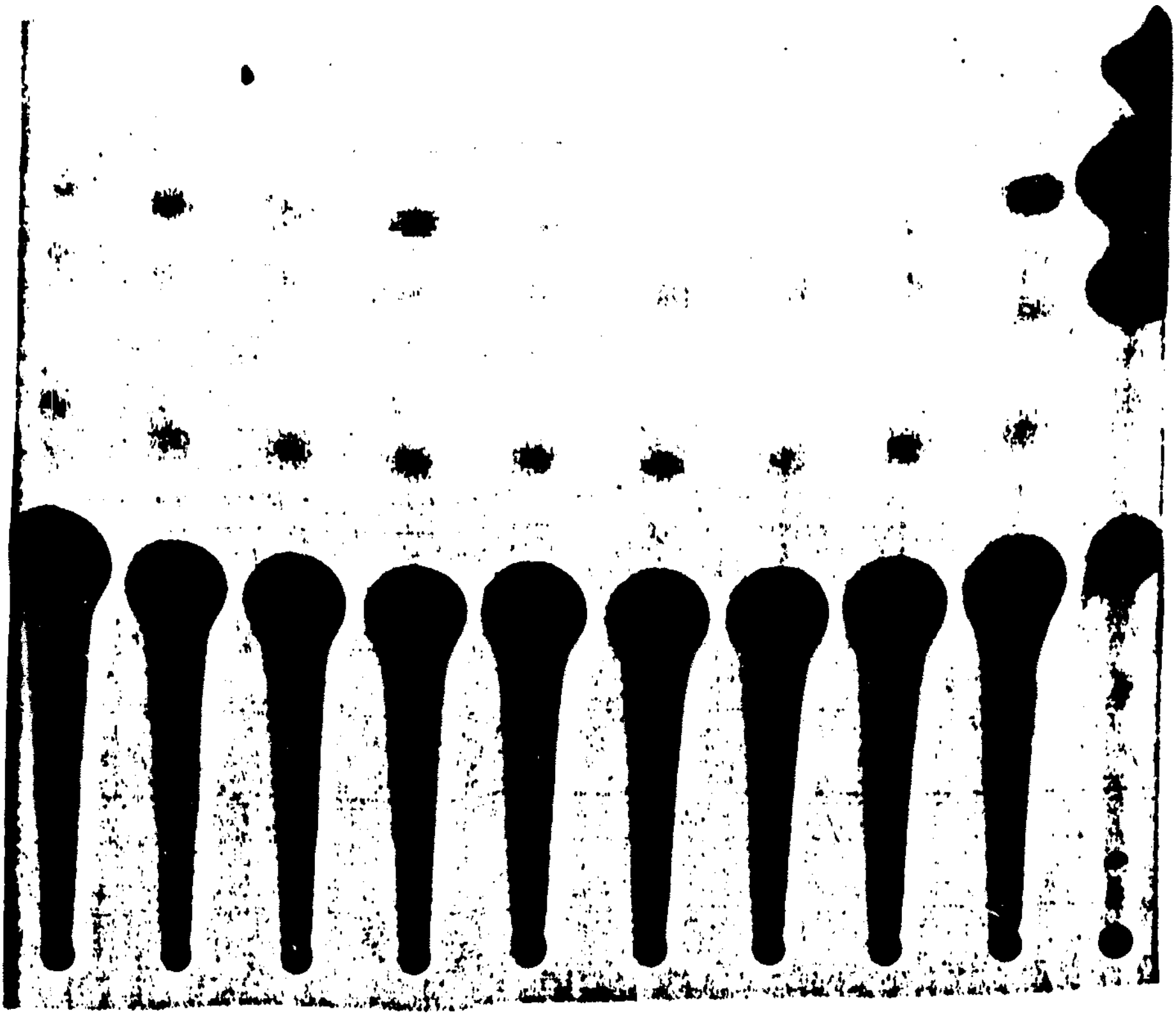


Figure 12. First series of CAT assays on fourth series of transfections (from left to right; negative control, pSVe-CAT, pSVe-CAT, pSVe-CAT, pSVe-CAT#24, pSVe-CAT#24, pSVe-CAT#68, pSVe-CAT#68, pSVe-CAT#68, positive control).

Figure 13. Second series of CAT assays on fourth series of transfections (from left to right; negative control, pSVe-CAT, pSVe-CAT, pSVe-CAT, pSVe-CAT#24, pSVe-CAT#24, pSVe-CAT#68, pSVe-CAT#68, pSVe-CAT#68, positive control).

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